STRUCTURE AND FUNCTION OF THE HUMAN

LACTATE DEHYDROGENASE VARIANT CALCUTTA-1

A thesis submitted for the degree of

Doctor of Philosophy

in the

Australian National University

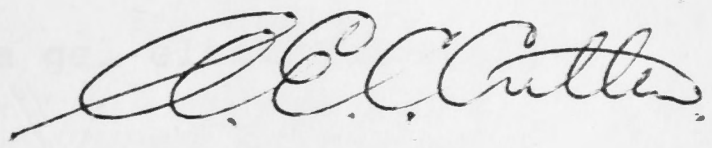
by

ANTHONY ELIOT CARGILL CUTTEN

John Curtin School of Medical Research

December, 1979

This thesis describes the results of research carried out in the Department of Human Biology, John Curtin School of Medical Research, Australian National University, Canberra, between January 1975 and March 1978 during the tenure of an Australian National University Research Scholarship. The results embodied in this thesis are my own work carried out under the supervision of Dr. R.L. Kirk except where they are otherwise acknowledged in the text. Additional research work was carried out in the Biochemistry Department, Sydney University, between November 1978 and November 1979.



Anthony E.C. Cutten.

Acknowledgements

I wish to acknowledge the advice and suggestions of my supervisor, Dr. R.L. Kirk, throughout this investigation.

I am grateful to Dr. J.V. Undevia, Dr. S.R. Das and Dr. A. Baxi for collecting biological material in Bombay and Calcutta, India.

The advice and assistance of Dr. H.A. McKenzie, Department of Physical Biochemistry and members of his group with physical and analytical measurements is gratefully acknowledged. In particular, Dr. D.C. Shaw willingly guided the peptide mapping experiments and with Mrs. N. Aldrich carried out the amino-acid analyses. Dr. R. Frier made the circular dichroism measurements and criticised the interpretation of the results. Dr. Koji Maeda assisted and advised on aspects of urea gel electrophoresis.

I am grateful to Dr. Gillian Air for helpful discussions on gel electrophoresis and protein analysis, to Dr. E.H. Creaser for advice on chromatographic techniques, to Dr. Elizabeth Heyde for discussions on kinetic experiments and to Dr. H.B. Younghusband for the gift of radio-labelled viral DNA.

Mr. N.M. Blake is thanked for his technical assistance only exceeded by his physical encouragement.

I wish to thank Dr. Bronya Keats for assistance with the computor programing and Mr. W. Craig and Mr. W. Clapton for advice on computor operational problems. Mr. R. Jones advised on statistical problems, for which I am grateful.

Mr. W. Hudson and Mr. S. Butterworth and members of the photography section were extremely helpful in the preparation of the photographs and figures in this thesis.

My thanks go to Ms. Elizabeth Robertson and to Ms. Jo Mahon for their patience and diligence with the typing of this thesis. I wish also to thank my brother, Mr. H. Cutten for collating the references.

Finally, I wish to acknowledge the award of an Australian National University Research Scholarship which provided the financial support for this project.

The author is grateful to Professors K.Taylor and G. Wake and to Dr. M.A. Thomas and Dr. G. Ralston of Sydney University Biochemistry Department for enabling further work to be carried out on this project.

Dr. C.A. Appleby of C.S.I.R.O. allowed me to use isoelectric focusing equipment and advised on the procedure. Dr. R.G. Whittaker of University of N.S.W. introduced me to the nanomole fingerprinting method. Their assistance is gratefully acknowledged.

Abstract

The human Lactate Dehydrogenase genetic variant Calcutta-1 is distributed widely in populations at frequencies of 1-4% across India. The LD_1 isoenzyme from normal erythrocytes, homozygous and heterozygous variant erythrocytes and the LD_5 isoenzyme from normal and heterozygous variant placentae were purified by ion-exchange and affinity chromatography to homogeneity.

1. Electrophoretic studies have shown that the enzyme variant is of the fast anodic B type. Two types of polypeptide chain were resolved on denaturing gels.

2. Thermostability studies suggest a thermosensitive mutation in the B subunit, manifest in the LDH of homozygous individuals, but not apparently affecting the LDH of heterozygous individuals.

3. Kinetic determinations on the purified isoenzymes suggested that the catalytic properties are little affected by the mutation. Affinity of the LD_1 variant enzyme for coenzyme is decreased.

4. Circular dichroism studies of the purified isoenzymes indicated that the secondary and tertiary structure have been conserved in Calcutta-1, however some evidence of possible conformational changes was obtained.

5. Amino-acid analysis and peptide mapping of the purified proteins was carried out, with a single difference peptide in the B subunit being tentatively identified.

The evidence of all the experimental techniques employed suggests that Calcutta-1 has very similar primary sequence, secondary structure, molecular size and kinetic properties to the normal enzyme. It was concluded that those differences observed between Calcutta-1 LDH and normal LDH were best explained by an amino-acid substitution in the B subunit, not in the A subunit as reported by earlier workers.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 3
(contd.)

LIST OF TABLES

1. INTRODUCTION

1.1 The biochemistry of LDH

Lactate dehydrogenase is a soluble glycolytic enzyme found in the cytoplasm of almost all animal tissues, in plants and in microorganisms. LDH's fall into two distinct groups based on electron donor: those which bind cytochromes or flavins and those which bind NAD. Characteristic of some lower animals and bacteria are a group of NAD-linked LDH's which reduce the substrate, pyruvate to the D isomer of lactate. This thesis is concerned with L-lactate, NAD-linked lactate dehydrogenase (E.C. 1.1.1.27) widely distributed in the animal kingdom.

LDH plays a central role in cellular carbohydrate metabolism. The enzyme reversibly catalyses the interconversion of lactate and pyruvate utilizing the redox couple NAD^+/NADH. During anaerobic glycolysis the enzyme catalyses the terminal step in the ATP-yielding breakdown of glucose, with L-lactate as product. In richly oxygenated tissues such as animal heart, lactate is oxidised by LDH to pyruvate which is further oxidised in the citric acid cycle yielding ATP.

The enzyme found in higher animals is polymeric, containing four subunits, each of approximately 35,000 M.W., resulting in a native protein of 140,000 M.W. The isoenzymic nature of LDH is apparent if a tissue homogenate from a vertebrate source is subjected to electrophoresis. Histochemical staining for the LDH activity characteristically results in the appearance

of five bands of activity regularly spaced along the zymogram. The spacing between the bands suggests a common increment in nett charge carried on each protein. Several lines of evidence have substantiated that each band represents a tetrameric protein made up of two types of subunit, A and B. The fastest (anodal) band, termed LD_1, comprises four B subunits (B_4), while the slowest band is LD_5(A_4). The three intermediate bands are the other possible combinations of A and B subunits; B_3A (LD_2), B_2A_2 (LD_3) and BA_3 (LD_4).

Appella and Markert (1961) and Cahn et al. (1962) demonstrated that treatment with 12M urea or 5M guanidine split each isoenzyme into four monomers. Wieland and Pfleiderer (1961) showed appreciably more aspartate residues (138 moles/mole of enzyme) were found in rat heart LD_1 than in rat liver LD_5 (118 moles/mole) while the LD_5 had more lysine residues than the LD_1 (100:89 respectively). Similarly a regular fall in aspartate plus glutamate content and a rise in lysine plus arginine content proceeding from LD_1 to LD_5 was demonstrated in the human isoenzymes (Wachsmuth et al., 1964). Wieland et al. (1964) showed that the fingerprint of LD_2 (B_3A) from pig had an appearance identical to that produced by a 3:1 mixture of pig LD_1 and LD_5. In a similar experiment it was shown by Wieland and Pfleiderer (1961) that LD_3 had an amino-acid composition almost exactly intermediate between that of LD_1 and LD_5.

Markert(1963) devised a convincing demonstration of the nature of the isoenzymes in what is now regarded as a classic experiment. The dissociation by freeze/thaw of a mixture of

equal amounts of beef LD_1 and LD_5, in 1M NaCl, gave a pattern on electrophoresis and staining of the five isoenzymes, 1:4:6:4:1 in intensity. This is just the pattern expected of the random reassociation of tetramers from fully dissociated A_4 and B_4 isoenzymes. Electrophoresis after similar treatment of each of LD_1 and LD_5 alone showed only a single isoenzyme.

1.2 The evolution of LDH

Early in the line of vertebrate evolution only a single gene encoded an A-like LDH polypeptide which assembled into a homotetramer with properties probably resembling those of the modern A_4 isoenzyme. This ancestral gene duplicated, and subsequent divergence by the acceptance of a series of mutations resulted in two distinct genes now recognized as A and B. Association between these gene products yields the five isoenzymes characteristic of mammals and birds. Lower vertebrates also possess the two genes, but unrestricted association between them is not always observed (Markert, et. al, 1975). In frogs and certain teleosts the B_4 isoenzyme does not hybridize with the A_4 isoenzyme *in vivo* or *in vitro* but the A enzyme will hybridize with mammalian and avian B_4 enzymes *in vitro*. An alteration in the frog B polypeptide has occurred which is incompatible with A subunit interaction, while the binding site of the A polypeptide for B subunits has been conserved.

The basic unit of LDH was defined early in the enzyme's evolution since a common subunit of M.W. 35,000 is found in all LDH's examined. In contrast to the tetrameric enzyme of higher animals, many bacteria and some lower animals possess a

dimeric D-lactate specific LDH of M.W. 70,000 again with 35,000 M.W. subunits. Even though the amino acid composition of LDH's from different species vary widely indicating the accumulation of many mutations, the basic subunit size has remained constant during evolution, with only minor differences in M.W. reported between species.

Structural and evolutionary advantage must be evident in the polymeric state since all the vertebrates have retained the tetrameric form of the enzyme. Protection against inactivation, perhaps by oxidation of the sulphydryl groups may result from tetrameric aggregation. Inherent in each subunit are the basic properties of the enzyme as a whole: a single active site per subunit binding one molecule of NAD and one molecule of substrate; the catalytic independence of each subunit (although cooperative effects between subunits have been reported for lobster tail LDH: Kaloustian and Kaplan, 1969); characteristics of the heteropolymers intermediate between those of the homopolymers in proportion to the A and B subunit content.

The evidence that A and B derived from a common ancestral form is several-fold: they share essentially the same tertiary structure from protein X-ray diffraction studies (Eventoff et al., 1975), an identical catalytic mechanism, a closely conserved dodeca-peptide sequence containing the "essential" cysteine 165 (Taylor et al., 1973), a common molecular weight determined by sedimentation (Nisselbaum and Bodansky, 1961), and in common with all LDH subunits, stereospecificity for the A side of the pyridine ring of NAD.

The divergence of B from A in the vertebrates is demonstrated in many ways including its accumulation of acidic residues in many species resulting in a fast anodal migration. Immunological cross reactivity was not observed between the B_4 isoenzyme and antiserum to the A_4 isoenzyme, nor in the reciprocal test (Markert and Appella, 1963; Markert and Holmes, 1969). Weak immunochemical relatedness between A and B was established however by methods considered to be of a higher degree of sensitivity (Holmes and Scopes, 1974). Many catalytic properties such a Michaelis constants, substrate optima, coenzyme analogue reactivity, thermal stability, susceptibility to inhibitors and binding and elution properties during chromatography distinguish A and B enzymes (Wilkinson and Withycombe, 1965; Nisselbaum and Bodansky, 1961). It has been suggested by Everse and Kaplan (1973) that A and B polypeptides differ by a minimum of 20% in amino-acid sequence.

Salmonid fishes demonstrate more than 15 isoenzymes of LDH. Evidence for duplication of both A and B loci to give A,A^1,B,B^1 gene products was found in cytological and biochemical studies (Morrison and Wright, 1966; Wunch and Goldberg, 1970). Quantitative immunological studies suggest structural divergence of B from B^1 over approximately 10^8 years (Bailey and Thye, 1975), since the duplication event. The salmonid B LDHs perhaps provide a model of how the A and B genes arose by duplication and divergence from a common ancestral form.

1.2.1 The C locus

Blanco and Zinkham (1963) and Goldberg (1963) discovered a third LDH locus in an investigation of human

testis which they called LDX. Additional LDH bands were found in the eye and brain of teleost fishes (Markert and Faulhaber, 1965) which were shown in the eye to be restricted to the retina (Nakano and Whitely, 1965). This eye specific LDH was named E_4LDH by Massaro and Markert (1968). Whitt (1969) used immunochemical methods to show that E subunits were more closely related to B subunits than A, while the finding of individual fish which were heterozygous for electrophoretic variants which only affected E subunit isoenzymes confirmed that E was an independent locus. The E_4 enzyme was shown to have kinetic and physical properties similar to those of B_4 (Whitt, 1970; Horowitz and Whitt, 1972), for example the pH optimum of both B_4 and E_4 was 7.2 while that of A_4 was 6.5. The E_4 enzyme had greater affinity for pyruvate and was more susceptible to substrate inhibition than either A_4 or B_4 isoenzymes. In those tissues where the E gene was expressed, E subunit appeared to exert a stabilising influence, directing the assembly of heteropolymers containing E, A and B subunits even though AB heteropolymers were not observed (Whitt, 1970).

Sensabaugh and Kaplan (1972) investigated a liver-specific LDH previously reported in teleosts by Odense et al. (1969). The liver specific enzyme, termed LDH F_4 was from Gadoid teleosts and was considered by the authors to originate from a distinct structural locus. Catalytic and immunochemical properties of F_4 demonstrated a relationship to the B_4 isoenzyme but it was distinct from both A_4 and B_4 isoenzymes in being more heat stable. In the same manner as the E_4

enzyme, liver specific enzyme hybridized with both A and B subunits which did not hybridize with each other *in vitro* or *in vivo*. The expression of specialised liver LDH appears to be confined to two orders of teleost, the Cypriniformes and the Gadiformes (Whitt *et al.*, 1975) generally exhibiting cathodal migration. The possession of an eye specific or a liver specific LDH appears to be mutually exclusive in the many teleost species examined by Whitt *et al.* (1973). In any particular species only one of the two possible lines of specialization appears to have occurred. The similarity between the properties of B_4, E_4 and F_4 LDH's of teleosts provides evidence for their homology and common evolutionary origin (Holmes, 1972) see Figure 1.1.

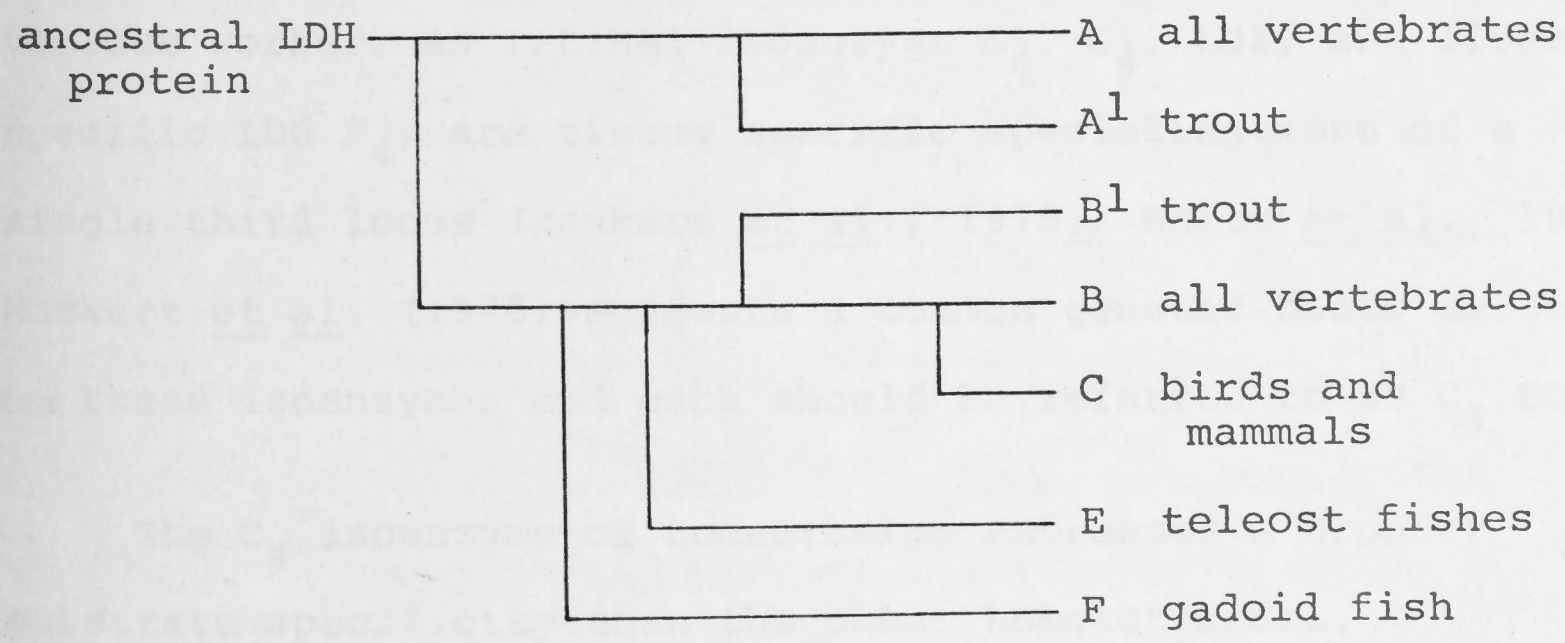


Figure 1.1 Proposed evolutionary relationship of LDH genes in vertebrates (after Holmes, 1972).

The testis specific LDX (C_4) present in the eutherian mammals during spermatogenesis was also found in two marsupials, the wombat and the wallaby (Baldwin and Temple-Smith, 1973) but was not found in monotreme (echidna and platypus) testis sampled at the peak of annual spermatogenesis. However, testis specific C_4 isoenzyme was reported in echidna as well as in many marsupial species by Holmes et al. (1973). An additional LDH band structurally and kinetically related to A_4 isoenzyme was found in the platypus by Baldwin (1973). The extra band, located in the LD_5 complex did not demonstrate the kinetic properties which characterise eutherian LDX and it was concluded that the band was an altered duplicate of the A gene and was not the product of a C locus.

It has been proposed that the LDH isoenzymes reported by various workers as retinal isoenzyme E_4, C_4, LDX, and liver specific LDH F_4, are tissue specific specializations of a single third locus (Markert et al., 1975; Whitt et al., 1975). Markert et al. (1975) suggests a common genetic basis exists for these isoenzymes and each should be referred to as C_4 LDH.

The C_4 isoenzyme of homeotherms expresses a broader substrate specificity than the other homotetramers, metabolizing a wide range of α-hydroxy acids not utilized by A_4 or B_4 isoenzymes. It is proposed (Whitt, 1969) that the C gene of birds and mammals was derived by duplication of the B locus. Zinkham et al. (1969) reported that the B and C loci are closely linked in pigeons and possibly

contiguous, suggesting tandem duplication. However, while B gene is expressed in nearly all tissues, C is precisely specific to the primary spermatocytes in sexually mature male homeotherms. Linkage therefore does not appear to prevent the independent regulation of the two genes.

A wide variation in electrophoretic mobility of the C_4 band from strongly anodal at pH 7 to a cathodal migration was observed (Markert et al., 1975). This variability contrasts with the stable structure and tissue expression of the A and B isoenzymes amongst higher vertebrates, placing C in an intermediate evolutionary position.

1.2.2 Structural homologies within the dehydrogenases

Another longer-term aspect of the evolution of LDH is provided by recent work on the structural homolgies within the dehydrogenase family of enzymes. As the detailed structures of intracellular enzymes become available from X-ray diffraction studies (Campbell et al., 1974; Stammers et al., 1977) it is becoming clear that a common structural feature is being shared by many different enzymes. The α-β structure, consisting of parallel strands forming a β-pleated sheet overlayed by α-helix inter-strand loops, was first observed in the dehydrogenases (Adams et al., 1970) and later in the kinases, was thought to be a common feature related to their nucleotide binding function. The dehydrogenases comprise a family of related, polymeric, NAD-linked proteins, including LDH, MDH, G3PDH, ADH and GDH, of which the LDH isoenzymes of

higher vertebrates form a single homologous sub-family. Each of the four related dehydrogenases ADH, MDH, LDH and G3PDH fold into two distinct domains.

One domain is responsible for binding NAD and is called the nucleotide binding fold (Blake, 1974), the other domain carries the site binding for the particular substrate and the catalytic site. In all four enzymes the nucleotide binding fold is characterised by a central core of β sheet composed of six parallel strands in the order CBADEF, linked by similar dispositions of α-helical interstrand loops above and below the plane of the β sheet - Figures 1.2.a,b,c.

The catalytic domains of each enzyme are structurally distinct with the exception of LDH and MDH which are closely similar (Blake, 1974). Rossman et al. (1974) proposed the hypothesis that the NAD binding domains of these enzymes evolved by divergence from a common ancestor and were incorporated by gene fusion with another fragment, characteristic of each enzyme which became responsible for the catalytic domain.

If both structural and sequence data together are used i.e. the superposing of hydrogen-bond arrangements in the β-sheet core of the compared enzymes, the homologous amino-acids are indicated. With the sequences thus aligned the dehydrogenases, flavodoxin and subtilisin demonstrate relalationships to each other. The relationships between these proteins are depicted in a evolutionary tree - Figure 1.2.d (Buehner et al., 1973).

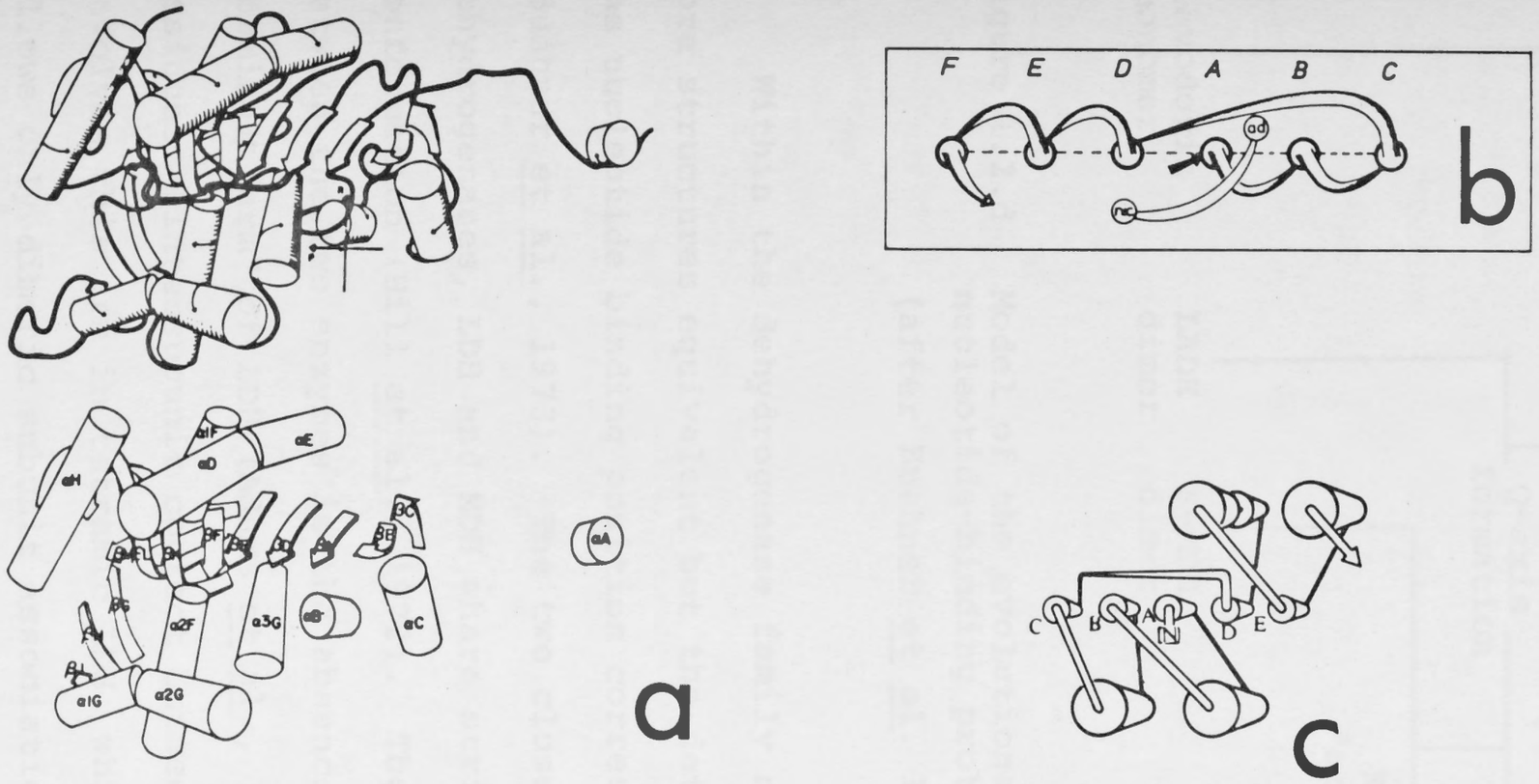


Figure 1.2. a. Model of the LDH subunit (after Rossmann et.al, 1974) b. NAD binding domain of LDH, MDH, ADH and G3PDH. Letters refer to the β strands (after Schulz and Schirmer, 1974). c. Structural domain common to LDH and PGM (after Campbell et al., 1974).

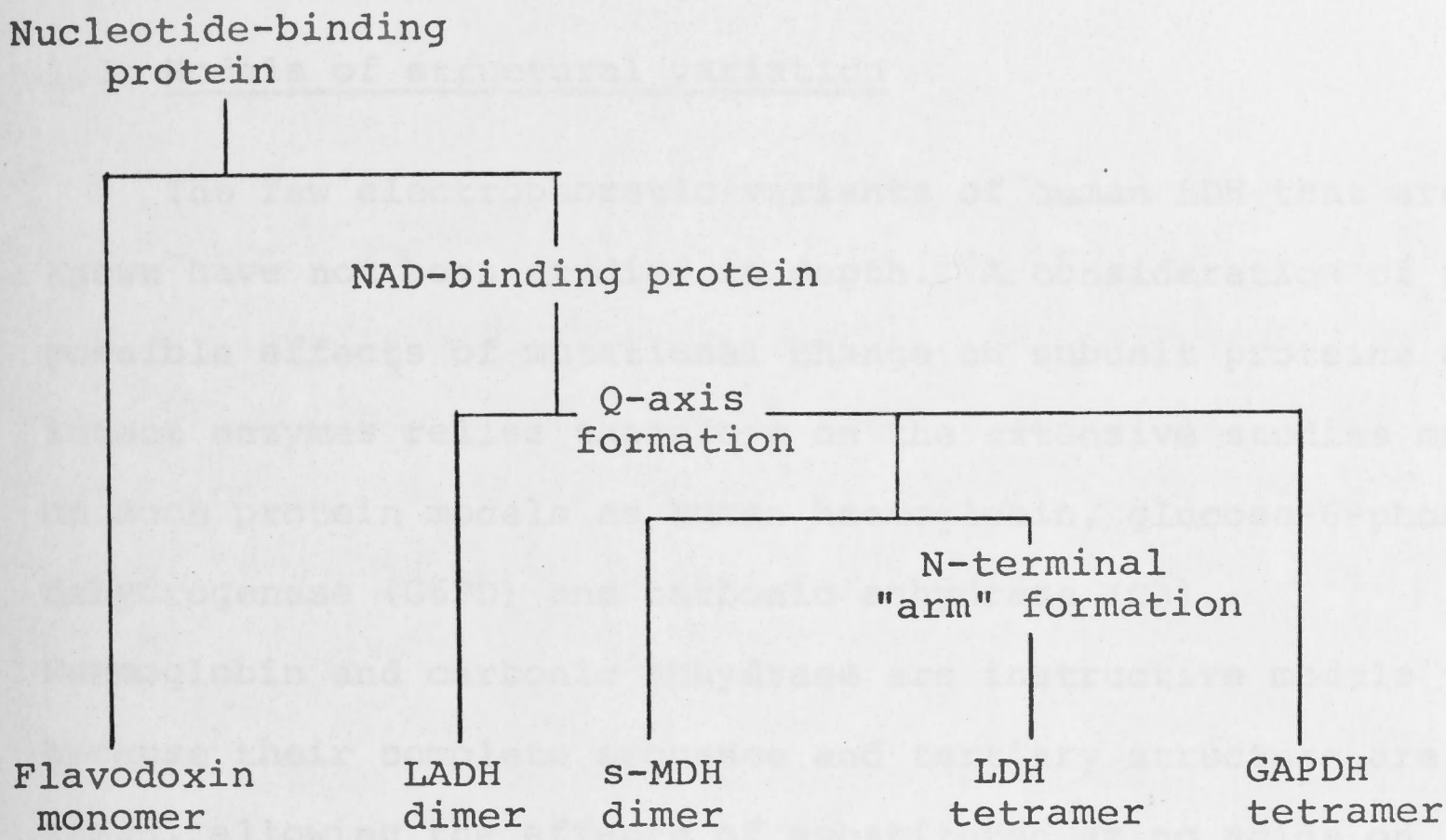


Figure 1.2.d Model of the evolutionary relationships of nucleotide-binding proteins.
(after Buehner et al. 1973).

Within the dehydrogenase family not only are the β sheet core structures equivalent but the detailed disposition of the nucleotide binding position corresponds between enzymes (Buehner et al., 1973). The two closely related dehydrogenases, LDH and MDH share strikingly similar structural conformation (Hill et al., 1972). The principal difference between the two enzymes is the absence in sMDH of the N terminal "arm" of LDH (Adams et al., 1970) consisting of 22 residues. Intersubunit contact between adjacent subunits involves this arm in tetramic LDH, while its absence in MDH allows only dimeric subunit association.

1.3 Models of structural variation

The few electrophoretic variants of human LDH that are known have not been studied in depth. A consideration of the possible effects of mutational change on subunit proteins and intact enzymes relies therefore on the extensive studies made on such protein models as human haemoglobin, glucose-6-phosphate dehydrogenase (G6PD) and carbonic anhydrase (CA).
Haemoglobin and carbonic anhydrase are instructive models because their complete sequence and tertiary structure are known, allowing the effects of substituted amino acids on neighbouring residues to be considered. G6PD is also considered here since as a nucleotide binding, subunit dehydrogenase it is functionally more closely related to LDH than the other proteins.

In common with LDH, haemoglobin is a tetrameric protein composed of two types of homologous subunit. However, whereas LDH subunits (at least in the higher vertebrates) will aggregate to form all possible tetrameric hybrids, haemoglobin chains associate preferably in the symmetrical interaction $\alpha_2\chi_2$, where $\chi = \beta, \gamma, \delta, \varepsilon$. These haemoglobin chains are homologous with each other and with myoglobin. It is proposed by Ingram (1962) that each of the chains arose by gene duplication from an ancestral gene related to myoglobin. The homotetramers β_4(HbH), γ_4(Hb-Barts) and ε_4(Hb Gower I) are observed in disease conditions - α-thalassemia, transitorily in the new born or in early foetal life respectively. The α_4 tetramer occurs in β-thalassemia but is very

unstable, precipitating to form inclusion bodies. The assymetric tetramers $\alpha\beta_3$, $\alpha_3\beta$ are not stable; a finding consistent with dissimilar and discriminating subunit contact sites. Lactate dehydrogenase however, strongly conserves equivalent binding sites on all subunits such that hybridization between A, B and C subunits occurs within almost all vertebrates *in vivo*, as well as interspecific hybridization *in vitro*.

More than 150 variants caused by amino-acid substitutions are documented in human haemoglobin (Masters and Holmes, 1975) and new variants are reported regularly in the literature, while so far only of the order of 20 are reported for human LDH. Variants of haemoglobin caused by mutational events other than by amino-acid substitution are also known. Many amino-acid substitutions cause minimal change in the structure and function of the haemoglobin molecule. The substitutions involving changes of chemical property cause most disruption to the structure, especially those involving polar or charged residues in internal areas of the protein (Perutz *et al.*, 1968). For example, Hb Hammersmith βCD1 Phe → Ser is one of the most unstable of haemoglobins. Phenylalanine makes apolar contact with the haeme group, but serine with a smaller polar side chain allows water into the normally hydrophobic haeme pocket. Hereditary methaemoglobinaemia is caused by the substitution of essential histidines which ligate Fe in the +2 state such that it can reversibly bind O_2. Examples of this type of altered haemoglobin are Hb M Boston αE7 His → Tyr and Hb M Iwate αF8 His → Tyr while some external substitutions

such as Hb Hotu βH4 Val → Glu, which causes disruption of the βH4 Val - βA11 Leu and βGH5 Phe apolar interaction, resulting in mild haemolytic anaemia, may cause disease, most are harmless. Only 8% of variants involving external substitutions are associated with disease and then usually only in the homozygous state e.g. HbS βA3 Glu → Val. Thus of the order of 90% of the variation of this type represents what are presumably acceptable mutations.

Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) gene is carried on the X chromosome in man. The enzyme is an NADP-linked dehydrogenase which oxidises only glucose-6-phosphate as an obligatory substrate. The native enzyme consists of two identical subunits of 55,000 M.W. in the presence of substrate and NADP but can form tetramers depending on the pH and ionic strength of the medium. More than 100 human, electrophoretic variants are known (Yoshida, 1975), some ten of which have altered substrate specificity. An anodically fast variant, present in about 18% of American Black males is designated G6PD-(A+). Yoshida (1967) found the amino-acid substitution Asn → Asp following purification and peptide mapping of this electrophoretic variant. In another variant, G6PD-Hektoen, Yoshida (1970) discovered the substitution His → Tyr by peptide mapping. The variant causes overproduction of the mutant chain resulting in four times the concentration of enzyme in the affected males. The substitution of histidine by tyrosine has been documented in four Hb variants in both α and β chains but is associated with a reduction in the concentration of mutant chains.

The enzyme carbonic anhydrase occurs as two homologous isoenzymes originating by tandem duplication of an ancestral form. Each isoenzyme is a single subunit of 30,000 M.W., 260 amino-acids and ligates a single zinc atom by three histidine residues. The so called "high activity" isoenzyme CA II is extensively distributed in the tissues of mammals including the erythrocytes, kidney, liver and brain while CA I is found in erythrocytes and kidney. Both isoenzymes have been sequenced, showing 60% homology, and fitted to high resolution 2 Å three dimensional structures (Notstrand et al., 1975). Carbonic anhydrase is an instructive model since in human populations, the amino-acid substitutions causing six of the twelve known CA I electrophoretic variants have been determined and located in the tertiary structure. Only in CA Ie Hull 225 Gln → Lys (Arg) does the substitution occur within the regular secondary structure, in helix G, where no disruption of the helix results. The remaining determined CA I variants are all external substitutions to basic charged residues. Although most mutant proteins have similar or lower thermostability to the normal protein, CA Id Michigan has an increased heat stability. It was suggested (Hewett-Emmett et al., 1976) that the substitution involved, 100 Thr → Lys located externally, might allow the formation of an ionic bond Lys 100 - Glu 102, so as to "tighten" the molecular structure. Supporting this possibility is the mutation of the residue 102 Glu → Lys in CA If London which results in lower heat stability (Osbourne and Tashian, 1974). The variant CA I Israel is an "activity variant" associated with a reduction in specific bicarbonate dehydrase

activity of 16 fold in the supposed homozygote. In 8 M urea the normal enzyme lost no activity, but the variant was labile under these conditions. Heated at 55°C for 60 min. the variant lost all activity but the normal enzyme lost only 15% of activity. Affected individuals who were apparently homozygous suffered renal tubular acidosis and nerve deafness. The variant enzyme had one less tyrosine than the normal (Shapiro et al., 1974).

1.4 Incidence and world distribution of human LDH variants

Genetic variants of LDH in humans demonstrable by electrophoretic methods are uncommon. Starch gel electrophoresis of haemolysates has been the most frequently utilized method for the detection of variants. The first B locus variant was detected in a Nigerian African (Boyer et al., 1963), while the first A variant was found in several members of a Brazilian family (Nance et al., 1963). An American survey of nearly one thousand hospital employees (both white and black) in the U.S.A. provided eight individuals whose LDH pattern of variation could be classified into one of four distinct types named Memphis-1, Memphis-2, Memphis-3, Memphis-4 (Kraus and Neely, 1964). All the variants were found in blacks except Memphis-4, which was found in a Caucasian and subsequently also in her family.

A slow A variant was reported in a white family in Britain (Davidson et al.,1965); later, a slow A variant appeared in an Icelandic survey (Mourant et al., 1968a).

Nilsson and Eriksson (1972) believe this is the same A variant as that reported earlier by Kraus and Neely as Memphis-4 in America. Subsequent population surveys have shown that LDH electrophoretic variants are particularly rare in Caucasians; no variants being detected, for example, in more than 7,000 persons from Scandinavian countries (Nilsson & Eriksson, 1972; Schneider et al., 1975). Variants have been found in West Africans but not in the Bantu of South Africa (Kirk, 1971). Several different LDH variants have been reported from Malaysia and Singapore (Blake et al., 1973a; Lie-Injo et al., 1973) in Malays, Chinese and Indians.

LDH variants appear to be absent in South American Indians (with the exception of one report by Kirk et al., 1974), and in Australian aboriginals, the Indonesian and the Papua New Guinea peoples (with the localised exception of the Enga-speakers, Blake et al., 1969). A summary of reports of variants and their estimated frequencies so far reported around the world is presented in Table 1.1. Surveys in which no LDH variants were detected are summarized in Table 1.2.

Table 1.1

WORLD DISTRIBUTION OF HUMAN LDH VARIANTS

AUTHORS	POPULATION	NO. INDIVIDUALS SCREENED	NO. VARIANTS	SUBUNIT & TYPE	NAME	VARIANT FREQUENCY
Boyer et al. (1963)	Nigeria, Yorumba tribe	200	1	B slow	(Memphis-3?)	0.5%
Nance et al. (1963)	Brazil	Family data	4	A		-
Kraus & Neely (1964)	U.S. Negroes	610	7	(A fast (A fast (B slow	Memphis-1) Memphis-2) Memphis-3)	1.1%
	U.S. Caucasians	330	1	A slow	Memphis-4	0.3%
Vessel (1965)	U.S. Negroes	600	3	A fast	(Memphis-1?)	0.5%
	U.S. Caucasians	600	1	B fast	(homozygote?)	0.17%
Tashian (unpublished)	U.S. Negroes	95	1	A	(unpublished, referred to in Vessell (1965)	1.05%
Davidson et al. (1965)	Britain, Caucasians	1015	2	A slow	LDH As (Memphis-4?)	
	Turkish Cypriots	245	2	A fast	LDH A_F (Memphis-1 or -2)	
	Nigerians(Ibadan)	23	1	A fast		
Mourant et al. (1968a)	Icelanders	1000	4	A slow	(Memphis-4?)	0.4%

Table 1.1 contd.

AUTHORS	POPULATION	NO. INDIVIDUALS SCREENED	NO. VARIANTS	SUBUNIT & TYPE	NAME	VARIANT FREQUENCEY
Blake et al. (1969)	NEW GUINEA Highland Enga Speakers	408	6	A slow	Memphis-4?	1.47%
Sinnett et al. (1970)	NEW GUINEA Highland Yandapu-Enga	416	6	A slow	Memphis-4?	1.44%
Das et al. (1970a)	INDIA Bengalis	First report of Calcutta-1, see Table 1.3				
	Madras Tamils	717	2	B slow	Madras-1	0.28%
Ananthakrishnan et al. (1970)	INDIA Tamil Nadu miscellaneous castes	365	2	B slow	Madras-1	0.58%
Herzog et al. (1970)	KENYA Luo tribe	474	1 1		Memphis-2?) Memphis-4?)	0.42%
Das et al. (1972b)	INDIA Bengali Muslims	Relatives	3	A fast	Calcutta-2	-
	various Delhi	500	1	B slow	Delhi-1	0.2%
Reys et al. (1972)	MOZAMBIQUE Bantus	270	1	? slow		0.37%

Table 1.1 contd.

AUTHORS	POPULATION	NO. INDIVIDUALS SCREENED	NO. VARIANTS	SUBUNIT & TYPE	NAME	VARIANT FREQUENCY
Nilsson et al. (1972)	Finns	1769	2	A slow		0.11%
Ananthakrishnan et al. (1972b)	Bulgaria	138	1	B slow		0.72%
Blake et al. (1973a)	SINGAPORE Malays	259	1	A fast	Malay-1(Calcutta-1?)	0.78%
			1	A fast	Malay-2	
	Chinese	378	2	A fast	Chinese-1)	0.79%
			1	A slow	Chinese-2)	
Lie-Injo et al. (1973)	MALAYSIA Malays	1030	2		CAL-1)	0.39%
			1		MAD-1)	
			1	A fast	Kuala Lumpur-1)	
	Indians	1189	12		CAL-1)	0.42%
			5		MAD-1)	
			1	A slow	KL-2)	
	Chinese	1075	2		KL-1)	0.37%
			1		KL-2)	
			1	B slow	KL-3)	
Blake et al. (1973b)	W.Caroline Is. (Fauralep Atoll)	382	1		CALCUTTA-1	0.26%

Table 1.1 contd.

AUTHOR	POPULATION	NO. INDIVIDUALS SCREENED	NO. VARIANTS	SUBUNIT & TYPE	NAME	VARIANT FREQUENCY
Das et al. (1973a)	INDIA Dhangars	999	3 (relatives)		DELHI-1	0.3%
Mukherjee et al. (1974)	INDIA Muslims (W.Bengal)	123	1	A fast	CALCUTTA-2	0.81%
Papiha et al. (1975)	BANGLADESH Bengali Muslims	200	1		CALCUTTA-1	0.5%
Hamilton et al. (1975)	JAPAN Hiroshima Nagasaki	3135	1	B fast	un-named	0.03%
Ueda et al. (1977)	JAPAN		1	B slow	TOKYO-1	-
Saha et al. (1976)	INDIA-KERALA various castes Muslims & Christians, tribal groups	980	2		MADRAS-1	0.20%
Neel et al. (1976)	MARSHALL IS. (Micronesia)	373	4		CALCUTTA-1?	1.07%

Table 1.1 contd.

AUTHORS	POPULATION	NO. INDIVIDUALS SCREENED	NO. VARIANTS	SUBUNIT & TYPES	NAME	VARIANT FREQUENCY
Godber et al. (1976)	TANZANIA Sandawe tribe	206	3	?-fast	un-named	1.45%
Kirk et al. (1977)	IRAN Caspian littoral people		1		CALCUTTA-1	

Table 1.2

POPULATION SURVEYS WHERE LDH TESTED, NO VARIANTS REPORTED

REGION	AUTHORS	COUNTRY	POPULATION	NO. INDIVIDUALS SCREENED
AMERICAS	Weitkamp et al.(1970)	Sth.Venezuela	Makiritare Indians	535
	Weitkamp et al.(1972)	Sth.Venezuela & Nth.Brazil	Yanomama Indians	2353
	Tanis et al.(1973)	Venezuela	Yanomama Indians	1525
	Mondiano et al.(1972)	Peru	Indians	275
	Kirk et al.(1974)	Colombia	Amerindians	294 (2 aberrant patterns)
	Vergnes et al.(1976)	Bolivia	Chipaya) Siriono) tribes	221
	Shih and Hsia (1969)	U.S.	Taiwanese Chinese	100
			Negroes & Caucasians	100
	McAlpine et al.(1974)	E.Canadian Arctic	Eskimos	362
S.E.ASIA/ AUSTRALIA	Kirk et al.(1972)	Australia	Queensland Aboriginals	620
	Kirk et al.(1973)	Indonesia	W.Irianese Dani	258

Table 1.2 contd.

REGION	AUTHORS	COUNTRY	POPULATION	NO. INDIVIDUALS SCREENED
S.E.ASIA/ AUSTRALIA contd.	McDermid et al.(1973)	Indonesia	Batak of Samosir Is. Sumatra	188
	Blake et al.(1973c)	Australia	Lebanese immigrants	147
	Lie-Injo et al.(1974)	Indonesia	Batak of Nth.Sumatra	502
	Malcolm et al.(1972)	New Guinea (Manus Is.)	Usia 45) Manus 138)	183
	Woodfield et al.(1974)	New Guinea/ Papua	Fuyuge speakers	173
	Ganesan et al. (1976)	East Malaysia	Dyaks (Ibans) of Sarawak	505
JAPAN	Omoto et al.(1972)	Japan	Ainu of Hokkaido	125
	Ueda et al.(1974)	Japan	Hiroshima Japanese	1300
SOUTH ASIA	Roberts et al.(1972)	Sri Lanka	Singhalese	156
	Mourant et al.(1968b)	Bhutan	Bhutanese	152
	Singh et al.(1974)	India	Punjabis	500

Table 1.2 contd.

REGION	AUTHORS	COUNTRY	POPULATION	NO. INDIVIDUALS SCREENED
EUROPE/ MIDDLE EAST	Nilsson et al.(1972)	Nth.Europe	Swedes 4171)	
			Icelanders 1000)	
			Lapps 1776)	7820
			Finn/Swedes 873)	
			Maris 317)	
		(N.W.Greenland)	Eskimos 153)	
	Welch et al.(1973)	Britain	Orkney Islanders	408
	Cartwright et al.(1976)	Britain	Holy Islanders (Northumberland)	122
	Farhud et al.(1973)	Iran	Persians	134
	Marengo-Rowe et al. (1974)	Sth.Arabia	Arabs	261
	Schneider et al.(1975)	Greece	Greeks	219
AFRICA	Kirk (1971)	Sth.Africa	Bantus	304

1.4.1 The distribution of Calcutta-1

The greatest incidence of genetic variants of LDH occurs in the Indian subcontinent. The first of these variants was reported by Das et al., (1970a). Ten examples of the same variant were found in a total of 614 Bengalis sampled in Calcutta (a variant frequency of 1.63%) and following convention was named Calcutta-1. Interest in the variant strengthened as further occurrences were reported. Das et al. (1970b) confirmed the presence of Calcutta-1 (Cal-1) variant in Bengal, both in Calcutta and in surrounding villages at levels of incidence around 2% - Table 1.3. Family studies indicated a simple Mendelian inheritance of the allele. The variant was not restricted to Eastern India since Blake et al. (1970) in examining 500 Gujaratis and over 500 Marathis in Bombay found 14 cases of Cal-1, 5 in Gujaratis and 9 in Marathis representing 1.00% and 1.79% of the samples respectively.

A later study (Undevia et al., 1972) reported the presence of the Cal-1 gene in a distinct religious group in Bombay. The Parsis and Zoroastrian Iranis are followers of the Zoroastrian religion. Parsis migrated from Persia to Gujarat in West India about 1,300 years ago, after the destruction of the Sassanian Empire by Muslim invaders and have maintained a large degree of social and cultural isolation since. The Iranis are derived from the remnants of the Zoroastrian communities in the Yezd area of Iran and migrated to West India only in the last 150 years. In a

sample of 418 from a population of about 100,000 Parsis, 15 individuals with Cal-1 were found, representing 3.59% of the sample, while the variant was absent in the sample of 48 Iranis from a total Irani population in Bombay of about 6,000 (Undevia et al., 1972).

Reports on populations in North India (Blake et al., 1971) and in South India at frequencies up to 4% (Ananthakrishnan et al., 1972a) extended the geographic range of known occurrence of Cal-1, and to several further important generalizations regarding the variant. First, that the lower frequency of the allele in northern populations, (Mukherjee et al., 1975a) or even its complete absence in the Punjab (Singh et al., 1974) and Bhutan (Mourant et al., 1968b) suggests an increasing incidence from north to south. Further statistical work might demonstrate whether this change in incidence represents a distribution cline. Second, that caste investigations have shown somewhat higher incidence of the gene in lower castes than in upper. For example Rajputs and scheduled castes showed frequencies of 2.44% and 2.35% respectively in Delhi collections (Blake et al., 1971) while Brahmins and Katris were 1.6% and 1.28% respectively. In South India, Nadar and miscellaneous castes (lower castes, aggregated due to small sample size) presented frequencies of 4.14% and 3.32% respectively, while the variant was absent in Reddiar, Thevar and only 0.93% in Brahmins (Ananthakrishnan et al., 1972a).

These data suggest a non-uniform though broad geographic spread of the allele through the modern Indian populations

(Kirk, 1973b) yet a long history within widely different groups in the subcontinent, such that the allele has crossed barriers of caste and religion. Calcutta-1 is not only found at high frequency in some Hindu people but within Muslims in West Bengal (Mukherjee et al., 1974) at an incidence of 2.43%. Within a tribal population, the Kadar of Kerala State, Saha et al. (1974a) reported a Cal-1 frequency of 3.29%.

With the widespread distribution of the LDH Cal-1 allele, frequently exceeding the 1% level, the existence of homozygotes for the variant might ~~not~~ be ~~un~~expected. Detection of homozygosity for a mutant allele is possible provided the zygote does not suffer severely lowered viability in utero or in early post-natal life. The first suspected homozygote for LDH^{Cal-1} was reported in a girl from Tamilnadu by Ananthakrishnan et al. (1970). In this case the isoenzyme bands on electrophoresis of haemolysate from the homozygous propositus, moved as singlets, each in advance of the corresponding normal control isoenzyme bands (Das et al., 1970a). A Muslim family from Kapadvanj village, Gujarat state provided two further cases of homozygotes in a brother and sister (Saha et al., 1974b). The pedigree showed that of the issue of each of these individuals, all the living offspring were found to be heterozygous for Cal-1. Each of the three Cal-1 homozygotes reported so far was a healthy adult, aged 19, 47 and 45 respectively, with no evidence of clinical symptoms or disease.

Isolated cases of Calcutta-1 have been found outside India. A single example has been noted from an individual living in the Western Caspian region of Iran (Kirk et al., 1977). This report is the only indication at present available that Calcutta-1 might possibly have originated from the Middle East. It is possibly more likely that the Parsis acquired the gene after their arrival in India. The Gujarati-speaking Parsis are interesting therefore as possessing more than twice the frequency of Cal-1 as the local Marathis and Gujarati-speaking Hindus in the Bombay region. Undevia et al. (1972) suggest that malarial selection may be a factor in selecting for Cal-1, leading to one of the highest frequencies reported in India.

In addition to the Caspian individual, Blake et al. (1973) found a single case in a 17 year old female on Fauralep Atoll in the Western Caroline Islands in the Pacific. Although it is conceivable that an independent mutation has occurred, introduction of the allele by miscegenation with a visitor, possibly an Indian sailor may have occurred. However, Neel et al.(1976) found four apparently unrelated individuals among Micronesians in the Marshall Islands, who exhibited what appeared to be the Cal-1 phenotype. The variant frequency was 1.07% in the sample of 373 individuals tested.

Table 1.3

INCIDENCE OF CALCUTTA-1 IN POPULATIONS IN INDIA

POPULATION GROUPING	NO. INDIVIDUALS SCREENED	NO. VARIANTS	VARIANT FREQUENCY	AUTHOR
CALCUTTA-1 VARIANT				
CALCUTTA Bengalis	614	10	1.63%	Das et al.(1970a)
CALCUTTA Bengalis	173	3	1.75%	Das et al.(1970b)
Village "	98	2	2.04%	" " " "
BOMBAY Marathi	504	9	1.79%	Blake et al.(1970)
" Gujarati	501	5	1.00%	" " " "
TAMIL Brahmin	323	3	0.93%	Ananthakrishnan et al. (1970)
" Naicker	168	5	2.98%	" " " "
" Nadar	145	6	4.14%	" " " "
" Reddiar	148	0	0	" " " "
" Other castes	365	11	3.32%	" " " "
" Non Hindu	22	0	0	" " " "
NORTH INDIA Arora	71	0	0	Blake et al. (1971)
Brahmin	60	1	1.64%	" " " "
Khatri	77	1	1.28%	" " " "
Rajput	40	1	2.44%	" " " "

Table 1.3 contd.

POPULATION GROUPING		NO. INDIVIDUALS SCREENED	NO.VARIANTS	VARIANT FREQUENCY	AUTHOR
NORTH INDIA contd.					
Vaish		62	1	1.59%	Blake et al.(1971)
Scheduled Castes		83	2	2.35%	" " " "
Misc. Hindu		32	0	0	" " " "
Misc. Non-Hindu		61	1	1.61%	" " " "
BENGAL					
Upper caste Hindus (Brahmins,Kayastha,Vaidya)		493	2	0.41%	Das et al. (1972a)
Lower caste Hindus		937	17	1.81%	" " " "
TAMIL NADU					
Upper caste Hindus		323	3	0.93%	" " " "
Lower caste Hindus		313	11	3.51%	" " " "
BOMBAY	Parsis	418	15	3.59%	Undevia et al.(1972)
	Iranis	48	0	0	" " " "
BOMBAY DHANGARS	Dange	68	1	1.47%	Das et al. (1973a)
sub groups	Halmat	14	0	0	" " " "
" "	Hande	98	1	1.02%	" " " "

Table 1.3 contd.

POPULATION GROUPING		NO.INDIVIDUALS SCREENED	NO.VARIANTS	VARIANT FREQUENCY	AUTHOR
BOMBAY DHANGARS contd.					
	Hatkar	239	4	1.67%	Das et al. (1973a)
	Hattikanan	28	0	0	" " " "
	Khutekar	22	0	0	" " " "
	Mendhe	169	3	1.78%	" " " "
	Sangar	60	0	0	" " " "
	Shegar	100	2	2.00%	" " " "
	Unikankan	48	0	0	" " " "
	Zade	153	5	3.27	" " " "
KERALA					
	Kadar	213	7	3.29%	Saha et al. (1974a)
BIHAR					
	Oraon (tribe)	134	3	2.24%	Mukherjee et al. (1975b)
BIHAR	Munda(tribe) (Christians)	250	4	1.60%	Mukherjee et al. (1975b)
	Munda non-Christians	142	2	1.41%	" " " "
BENGAL	Mahishyas (Hindu)	100	1	1.00%	Mukherjee et al. (1974)
	Muslims	123	3	2.43%	" " " "

Table 1.3 contd.

POPULATION GROUPING		NO.INDIVIDUALS SCREENED	NO.VARIANTS	VARIANT FREQUENCY	AUTHOR
DELHI	Rajput	95	1	1.05%	Das et al. (1973b)
	Jath	104	1	0.96%	" " " "
	Gujjar	100	1	1.00%	" " " "
	Ahir	101	1	0.99%	" " " "
	Chamar	100	2	2.00%	" " " "
KERALA	Brahmin	60	0	0	Saha et al. (1976)
	Nayar	142	3	2.1%	" " " "
	Izhava	68	2	2.9%	" " " "
	Scheduled Castes	49	0	0	" " " "
	Muslim	125	1	0.8%	" " " "
	Christians	161	0	0	" " " "
	Malayarayan	59	0	0	" " " "
	Kurumba	43	3	6.9%	" " " "
	Toda	98	0	0	" " " "
TAMIL NADU					
	Irula	175	1	0.6%	" " " "
MAHARASHTRA					
	Brahmin	49	0	0	Mukherjee et al. (1975c)

Table 1.3 contd.

POPULATION GROUPING	NO.INDIVIDUAL SCREENED	NO.VARIANTS	VARIANT FREQUENCY	AUTHOR
MAHARASHTRA contd.				
Maratha	209	0	0	Mukherjee et al. (1975c)
Middle Castes	47	0	0	" " " "
Nava Budhas	144	3	2.08%	" " " "
Low Castes	41	0	0	" " " "
Muslims	55	2	3.64%	" " " "
Others	90	1	1.11%	" " " "
PUNJAB				
Brahmin	106	0	0	Singh et al. (1974)
Khattri	131	0	0	" " " "
Arora	103	0	0	" " " "
Jat	149	0	0	" " " "
PUNJAB				
Hindus (Punjabis, Khutri & Arora)	182	0	0	Mukherjee et al. (1975a)

1.5 The electrophoretic appearance of the Calcutta-1 phenotype.

Certain features of the banding pattern of the LDH isoenzymes from Calcutta-1 after electrophoresis in starch gel indicate that the variant has unusual properties.

Individuals who are heterozygous for other LDH variants reported have patterns to be expected for mutations affecting the charge on either the A or B subunit. Heterozygotes for either the A or B locus will possess two types of A or B polypeptide. Random association of these subunits should give rise to multiple banded patterns (Shaw and Barto, 1963; Koen and Goodman, 1969). For a B locus mutation, giving rise to a β subunit, five bands at the LD_1 position result from all possible tetrameric associations of B and β. An example of the pattern observed when the anodic mobility of β exceeds that of B subunit is shown in Figure 1.3.C sample 1. Provided that the B and β chains are synthesised in equal amount the relative proportions of the tetramers should be distributed binomially, $[B + \beta]^4$; 1:4:6:4:1 respectively. In the tetrameric isoenzyme model now generally accepted for LDH isoenzymes, structural alterations in the B polypeptide would not be expected to affect the A polypeptide and the A_4 isoenzyme should appear as a single band of normal migration.

A reversal of this pattern should be observed for a mutation in the A locus producing α polypeptide. Five bands are expected in the LD_5, 4 at LD_4, 3 at LD_3, 2 at LD_2

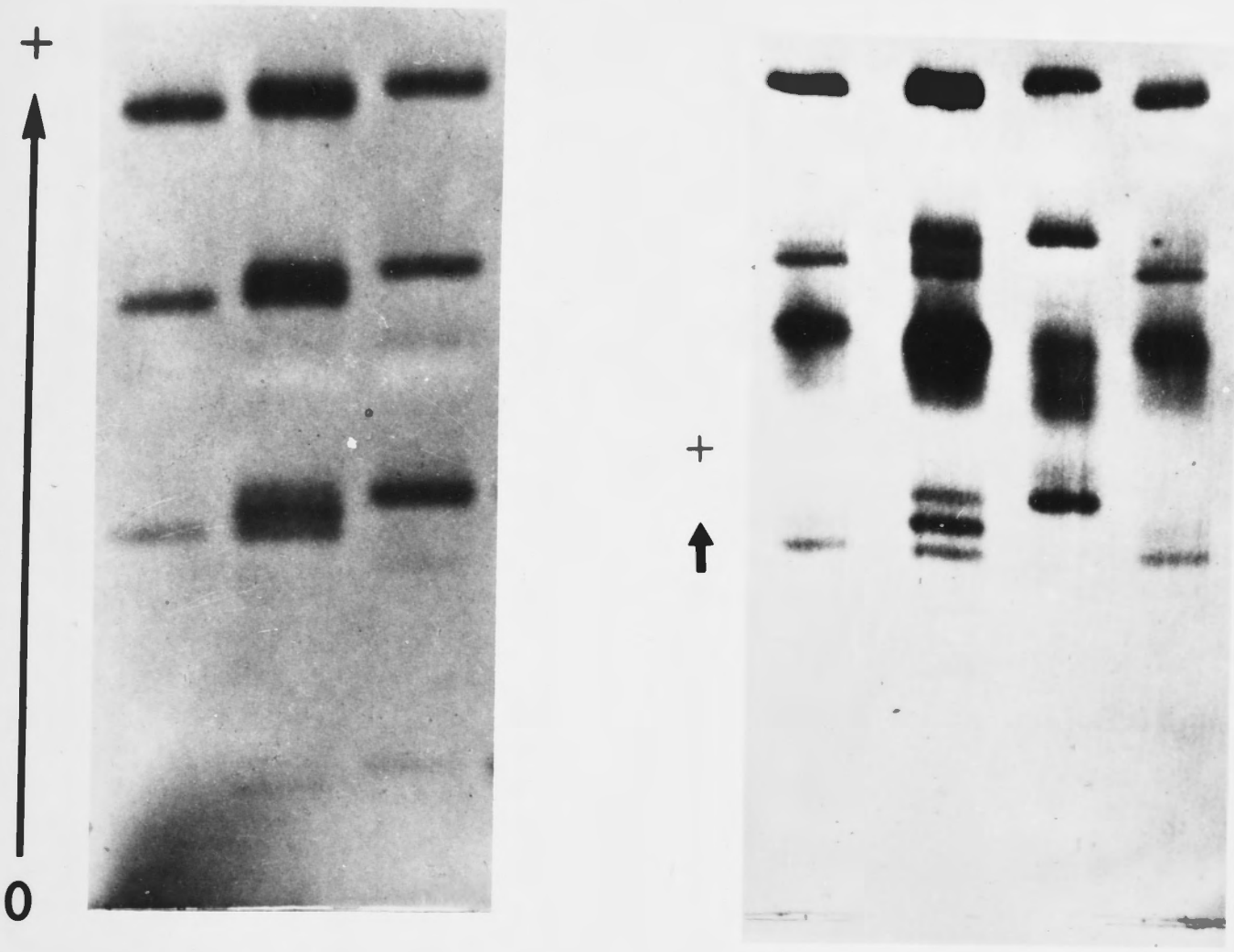


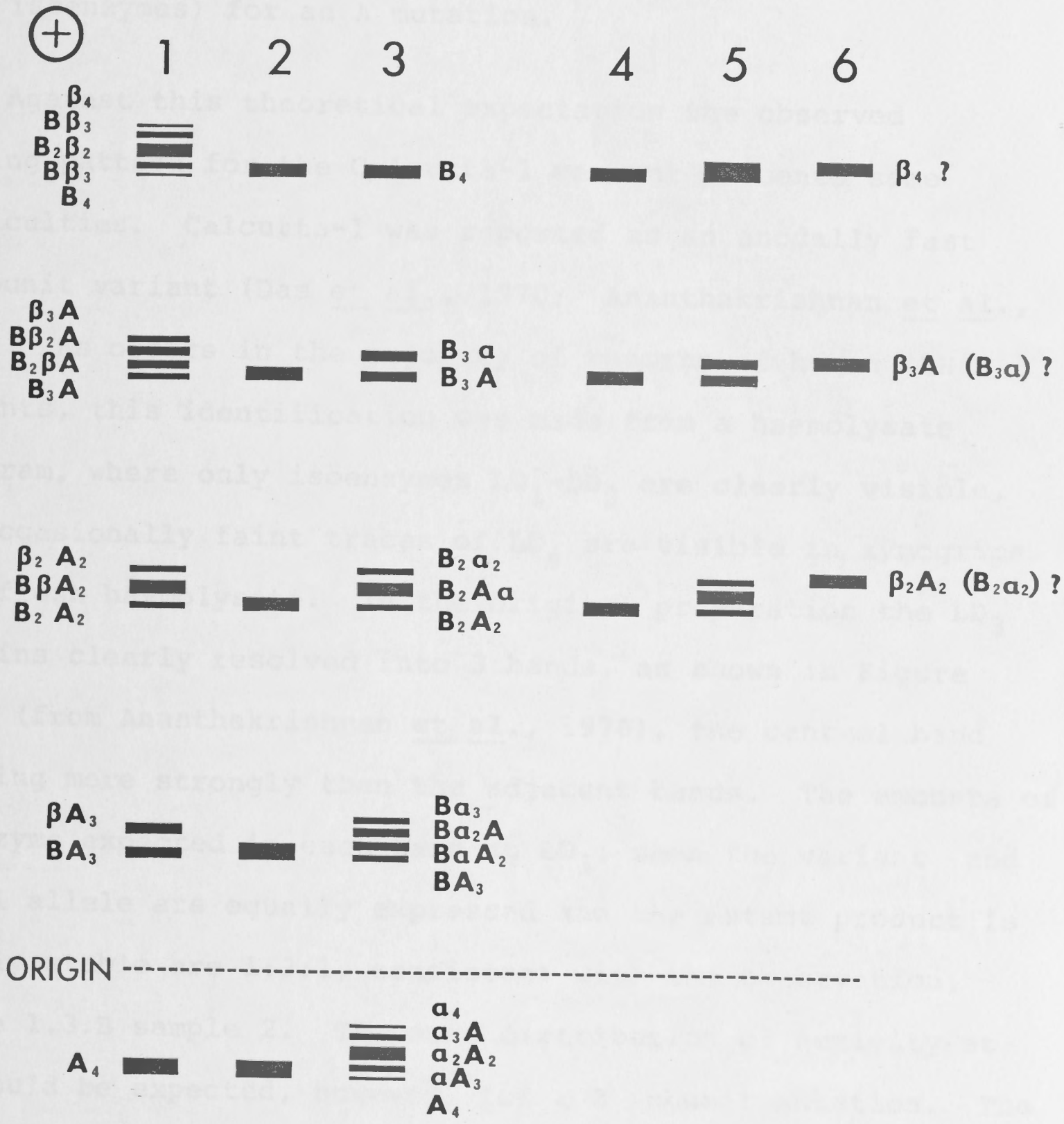
FIGURE 1.3

A. LDH zymogram, phosphate citrate buffer, pH 7.0.
a. Normal control; b. Calcutta-1 heterozygote;
c. Calcutta-1 homozygote. (from Saha et al., 1974;
reproduced with the permission of Dr R.L. Kirk.)

B. LDH zymogram, Tris/EDTA/borate buffer, pH 8.6.
1. and 4. normal controls; 2. Calcutta-1 heterozygote;
3. Calcutta-1 homozygote. (from Ananthakrishnan et al.,
1970; reproduced with the permission of Dr R.L.Kirk.)

Figure 1.3.c Variant LDH banding patterns on starch gel.

Sample 1. Fast B locus variant.
2. Normal.
3. Fast A locus variant.
4. Normal, erythrocyte.
5. Calcutta heterozygote erythrocyte.
6. Calcutta homozygote erythrocyte.


⊕
1
2
3
4
5
6
β4
Bβ3
B2β2
Bβ3
B4
B4
β4 ?
β3A
Bβ2A
B2βA
B3A
B3α
B3A
β3A (B3α) ?
β2 A2
Bβ A2
B2 A2
B2α2
B2Aα
B2A2
β2A2 (B2α2) ?
βA3
BA3
Bα3
Bα2A
BαA2
BA3
ORIGIN
A4
α4
α3A
α2A2
αA3
A4

with a single normal B_4 band at the LD_1 position. Figure 1.3.C sample 3 depicts the heterozygote variant pattern expected of a tissue such as placenta (which demonstrates all five isoenzymes) for an A mutation.

Against this theoretical expectation the observed banding pattern for the Calcutta-1 variant presents some difficulties. Calcutta-1 was reported as an anodally fast A subunit variant (Das et al., 1970; Ananthakrishnan et al., 1970). As occurs in the majority of reports of human LDH variants, this identification was made from a haemolysate zymogram, where only isoenzymes LD_1-LD_3 are clearly visible, and occasionally faint traces of LD_4 are visible in zymograms from fresh haemolysate. In the original preparation the LD_3 proteins clearly resolved into 3 bands, as shown in Figure 1.3.B (from Ananthakrishnan et al., 1970), the central band staining more strongly than the adjacent bands. The amounts of isoenzyme expected in each band at LD_3, when the variant and normal allele are equally expressed and the mutant product is equally stable are 1:2:1, consistent with the observation, Figure 1.3.B sample 2. The same distribution of activity at LD_3 would be expected, however, for a B subunit mutation. The pattern observed at LD_3 is equivocal then for Calcutta-1 being either a fast A mutation or a fast B mutation. At the LD_2 position there appear to be only two bands but they do not clearly separate. A broad 'band' in the LD_1 position has a trailing edge which aligns with the B_4 band of the homozygote normal. The leading portion of the heterozygote LD_1 'band'

is clearly anodal to the normal B_4 in both Figures 1.3.A and B, and in two different buffer systems: tris/EDTA/borate, pH 8.6; citrate/phosphate, pH 7.0. Furthermore haemolysate from the homozygote variant displays a single LD_1 band, with the same migration as the leading edge of the broad heterozygote LD_1 'band' and clearly faster anodally than the normal B_4. A single fast band is also seen at LD_2 and at LD_3 in the homozygote aligned with the faster and fastest heterozygote bands respectively - see Figure 1.3.B sample 3.

A problem posed by the identification of Calcutta-1 as an A variant is the nature of the behaviour of the LD_1 band. The B_4 type band should be unaffected by a mutation at the A locus. If Calcutta-1 is a B variant then it is of an unusual nature since the separation of bands at LD_3, (A_2B_2) is greater than at LD_1 (B_4). Since β should be more negatively charged than B, the separation between β_4 and B_4 bands should be twice as great as between β_2A_2 and B_2A_2 bands. The reverse of this expectation is observed.

In summary, the observation at LD_1 is indicative of a fast B mutation with band merging. However, the observation at LD_2 suggests a fast A mutation, but the possibility of the incomplete representation of the four expected bands of a fast B mutation is not excluded. The situation at LD_3 is exactly equivocal for either mutation. Taking into consideration the rare nature of observed mutation at either A locus or B locus, the probability of acceptance of mutations concomitantly at both loci is exceedingly remote.

LDH Calcutta-1 stands as the most successful of currently known LDH variants with respect to its penetration of a human population. This genetic success of the mutant directs enquiry toward the biochemical nature of the alteration and its effects. The studies reported here, therefore, attempt to resolve the following problems.

1. Is Calcutta-1 an A subunit or a B subunit mutation? How can the electrophoretic appearance of the isoenzyme bands be reconciled by the answer.
2. Is the nature of the mutation explained by an amino-acid substitution?
3. To what extent does the mutation affect the basic catalytic properties of the enzyme?
4. Are the thermodynamic properties of the enzyme affected?
5. Can anything be said about a possible site of mutation?

2. THE ISOLATION AND PURIFICATION OF HUMAN LDH

Lactate dehydrogenase activity is found in many tissues of the human body. The relative proportions of each of the isoenzymes is characteristic of each particular tissue (Wilkinson, 1965). Tissues such as heart, kidney and erythrocytes are rich in B subunit isoenzymes and prove a suitable source of B_4 homopolymer. In tissues like liver, muscle and placenta, the A subunit isoenzyme are preponderant. An important consideration therefore, in obtaining a high yield of the particular isoenzyme of interest, lies in the choice of a suitable tissue as source material. In the absence of cadaver or biopsy material only blood and placentae are readily available as sources of human LDH.

2.1 Earlier studies on the purification of LDH

LD_1 (B_4) has been commonly purified from heart muscle (Nisselbaum & Bodansky, 1963; Wachsmuth & Pfleiderer, 1963; Washsmuth *et al.*,1964 and Clark *et al.* 1976) or from erythrocytes, (Nisselbaum & Bodansky, 1963). The LD_1 proportion of total LDH activity in human heart muscle has been estimated at 73%, Vessel & Bearn (1961); 67%, Plagemann *et al.* (1960) and 49%, Wroblewski & Gregory (1961).

Erythrocytes are probably the next richest source of the isoenzyme, possessing 39%, Plagemann *et al.* (1960); 43%, Vessel & Bearn (1961) and 42%, Withycombe (1965) according to these workers.

Skeletal muscle or liver, Wachsmuth et al. (1964); Clark et al. (1976) and Nisselbaum & Bodansky (1963), were used as sources of LD_5 (A_4) for purification. Wroblewski & Gregory (1961) found liver had 81% LD_5 activity and Wieland et al. (1962) 80%, while the estimates of other authors are much lower e.g. Vessel & Bearn (1961), 36%. Skeletal muscle possesses a similar isoenzyme distribution to liver: 73% LD_5 Vessel & Bearn (1961); 78% Wieland et al. (1962) and 36% Wroblewski & Gregory (1961). Placentae demonstrated 25% LD_5 activity, Wieme & Van Maercke (1961), but individual placentae vary in their proportion of LD_5, (Blake, personal communication) and this figure may be an underestimate.

The earlier purification procedures of Nisselbaum & Bodansky (1963) and Vessel & Bearn (1961) relied on salting out techniques with ammonium sulphate, acid precipitation at pH 5.2, ethanol precipitation, adsorption to calcium phosphate gel and elution with 0.2M phosphate buffer and hydroxylapatite column chromatography. Nisselbaum & Bodansky (1963), purifying LD_2 from 500 ml of packed red cells achieved approximately 3,000 fold purification and a yield of 14% in 9 steps of purification. A similar procedure was utilized by Okabe et al. (1968) in purifying LD_3 from human uterine muscle with a 6% yield and 480 fold purification. Wachsmuth & Pfleiderer (1963) purified LDH from 2.5 kg of brain tissue using very similar techniques but omitted the ethanol extraction and hydroxylapatite and substituted a heat precipitation step (65^{o}C for 3 min.). DEAE-Sephadex column chromatography was used for the first time in LDH purification by these workers. The isoenzymes were eluted in a gradient

of from 0-0.45M NaCl in 0.033M phosphate buffer pH 7.2. The yield of total LDH was approximately 20% and purification 480 fold, prior to partitioning the isoenzymes on DEAE-Sephadex.

Pesce et al. (1967) demonstrated that essentially the same methods could be generally applied to the purification of LDH from a wide variety of vertebrates when they purified LD_1 from heart tissue of 4 species (including human) and LD_5 from the livers of 6 species of birds, fish, amphibia and mammals. Essential to all these purifications was the use of ammonium sulphate precipitations, varying selectively in the different species within the range of 30%-70% saturation. Some preparations were stable to acetone fractionation, but this method was not employed in the LD_5 purification. Ion-exchange chromatography was invariably used, DEAE-cellulose more commonly than CM-cellulose. Homogeneous protein preparations were achieved by these authors at 25-100 fold purification. Human heart LD_1 for example was purified in 6 steps from 9 kg of starting material, at a final yield of 30%.

Holmes & Scopes (1974) have also made use of cellulose ion-exchange resin, both CM or DEAE or both to purify A_4 and B_4 isoenzymes from a number of mammals, fish and bird sources.

The DEAE-Sephadex A50 column chromatography was further developed by Fritz et al. (1970), using a gradient of 0-0.22M NaCl in 20mM tris pH 7.4, to elute the isoenzymes.

The tissues used were rat heart and rat skeletal muscle. Isoenzyme 5 was unrestrained by the resin under these conditions and eluted near the void volume, while LD_1 eluted at 0.22M NaCl. The intermediate isoenzyme eluted sequentially as the B subunit content increased. Clark et al. (1976) scaled the same procedure down to 3 ml columns and proved the system was applicable to the rapid purification of small amounts of the human LDH's. A stepwise elution procedure replaced the gradient.

A recent technique which has become widely accepted as a purification method and has been applied to purifying LDH is affinity chromatography. Affinity chromatography utilizes adsorbent materials based on biological specificity.

The immobilization of ligands such as enzyme substrates, coenzymes or their structurally related analogues, to porous support materials has made possible the reversible, biospecific adsorption of enzymes, proteins and other macromolecules from solutions. This general subject has been reviewed by Cuatrecasas and Anfinsen (1971).

The purification of LDH from human placenta on amino-hexyl Sepharose linked oxamate derivative was achieved by Ocarra & Barry (1972) in the presence of NADH. The enzyme eluted following withdrawal of NADH from the irrigating buffer. An alternative approach has been the use of immobilized AMP-analogue which "mimics" the AMP moeity of NAD and is thus recognized by dehydrogenases which utilise NAD as coenzyme. Isoenzymes of beef LDH were separated by affinity chromatography on Amino-hexyl Sepharose bound AMP-analogue, eluting with a relatively weak NADH gradient (Brodelius & Mosbach (1973).

Ryan and Vestling (1974) observed that LDH co-eluted with marker Blue Dextran from a Sephadex G200 column, a result unexpected in view of their respective molecular weights, 140,000 and 2,000,000. These authors covalently linked Blue Dextran to Sepharose-4B forming a new affinity material. Using rat liver homogenate, LD_5 was eluted with low concentration NADH and recovered in excellent yield and purity. Blue Dextran affinity chromatography was further developed by Nadal-Ginard & Markert (1975) to purify LDH isoenzymes from a variety of vertebrate sources.

2.2 Extraction and purification of human LDH

In the present work several human tissues were purified by the methods described, especially in developing techniques such as the Blue Dextran affinity chromatography. In purifying LD_1, initially from human heart, methods such as acid precipitation, heat precipitation and calcium phosphate gel adsorption were used. Although they proved successful as early steps to remove large amounts of non-LDH protein, they were abandoned in favour of the gentler techniques of ion-exchange and affinity chromatography. Skeletal muscle and liver were used to yield large amounts of pure LD_5. Since erythrocytes and placenta containing variant enzyme were available, methods suitable to those tissues were developed.

2.2.1 Materials and methods

1. Biological. Whole blood was obtained from the Blood Bank and fresh human placentae were obtained from the Labour Ward, Canberra Hospital. Human liver and heart were obtained by cadaver autopsy, and skeletal muscle by surgical limb

amputation and resection collected from Woden Valley Hospital, Canberra. This material was determined as displaying normal LDH phenotype by starch gel electrophoresis.

The variant LDH bloods used in this investigation were collected in the course of population genetics studies, within India. Together with small survey samples of heterozygote Calcutta-1 variant haemolysate of various series, e.g. TPC, UND, NDH; a unit (400 ml) of whole blood was collected in Bombay by Dr. A. Baxi. The Cal-1 homozygote blood was an 80 ml (packed cell volume) sample collected in Bombay by Dr. J.V. Undevia. A single Cal-1 heterozygote placenta was obtained by Dr. S.R. Das from a maternity hospital in Calcutta and transported to Canberra over dry ice and stored under liquid nitrogen.

2. Chemicals. Details of chemicals used appear in Appendix-5.

Protein assay. Protein estimations of the preparation during the purification stages were made by the method of Kalckar (1947) using the equation ...

$$[\text{PROTEIN}]\ \text{mg/ml} = (1.55\ A_{280} - 0.76\ A_{260}) \times \text{dilution factor}$$

the equation ...

$$[\text{PROTEIN}]\ \mu\text{g/ml} = (A_{215} - A_{225})144 \times \text{dilution factor}$$

was utilized for the estimation of protein in more dilute solutions. Purified LDH protein was measured at 280 nm and the concentration calculated from the value ...

$$E_{280}^{0.1\%} = 1.35 \pm 0.06$$ after Pesce et al. (1964)

Kinetic assay. Details of the kinetic assay method used appear in Section 5.2.1.

2.2.2 Extraction

Washed red blood cells, stored under liquid nitrogen, were slowly thawed to 4°C and lysed in 20 mM phosphate buffer, pH 7.0, containing 3% w/v NaCl and 1mM β mercaptoethanol (βME). Extracts of human placenta, heart, liver or skeletal muscle were made by dicing the thawed tissue, adding 20 mM phosphate buffer, pH 7.0, 1 mM βME to 33% w/v concentration and homogenizing in a Sorvall Omnimix at 4°C. The haemolysate or tissue extracts were centrifuged at 10,000 g, 4-8°C for 30 min. and the supernatants estimated for protein and LDH activity. The precipitates of the solid tissues were re-extracted with a further volume of buffer, centrifuged and the supernatants combined with the earlier extractant and concentrated as necessary.

2.2.3 Purification methods

Ammonium sulphate fractionation

Pilot studies showed that LDH precipitated between 35% and 65% w/v ammonium sulphate concentration.

Tissue preparations and haemolysates were made 35% of sat. in $(NH_4)_2SO_4$ by slow addition of the solid with constant stirring, allowed to stand overnight at 4°C and centrifuged at 10,000 g for 30 min. The residue was discarded and the concentration of $(NH_4)_2SO_4$ in the supernatant was increased to 65% of sat. by the gradual addition of crystalline solid, with stirring in the cold and allowed to salt out for 3 hr. before centrifugation. The precipitate was removed, dialysed against at least 4 changes of phosphate buffer, each of 5 litres, and concentrated.

2.2.4 DEAE-Sephadex chromatography

DEAE-Sephadex A50 anion exchange resin, Pharmacia, was swollen overnight at 4°C against 20 mM tris/HCl pH 7.4 containing 1mM dithiothreitol (equilibration buffer). After removing the fines in a large measuring cylinder, columns were packed under gravity, as described in the Sephadex Technical Bulletin. Flow rates were checked and equilibration ensured by measuring the pH of eluate after passing 5-10 column volumes of equilibration buffer through the column.

Preliminary separation of the isoenzymes

Method 1. Sample, dialysed against equilibration buffer, was applied to the column in a small volume. After pumping 2 column volumes of buffer, a linear gradient of 0-0.25 M NaCl in buffer was applied. Fractions of 15 ml were collected at 30 ml/hr from the 30 cm × 2 cm column (flow 9.5 $ml/cm^2/hr$) and assayed for LDH activity and for protein.

A large peak of protein eluted from the column immediately following the void volume, containing what proved after concentration and starch gel electrophoresis, to be LD_5. A series of peaks of activity were eluted by the gradient, as depicted in Figure 2.1, in the order LD_4, LD_3, LD_2, LD_1 (when the preparation contained all of the isoenzymes).

LD_1 preparation

Method 2. Dialysed sample was applied and the development of the column with 2 column volumes of 0.16 M NaCl in buffer was commenced immediately. Three column volumes of 0.22 M NaCl in buffer followed. This procedure was repeated on a fresh column using as sample the broad peak of activity eluted by 0.22 M NaCl diluted with an equal volume of equilibration buffer.

Elution with 0.16 M NaCl resulted in a peak of activity including (LD_5), LD_4, LD_3 and (LD_2) in proportions depending on the tissue source - Figure 2.2. Final elution with 0.22 M NaCl gave a peak of LD_1 activity containing LD_2 and LD_3. With the second column - Figure 2.3, elution with 0.18 M NaCl removed LD_2 and LD_3 together with a small amount of LD_1. The major portion of LD_1 was eluted with 0.22 M NaCl free of other activity.

The procedure is summarised in the following flow-chart and the resulting elution profiles and zymograms are depicted in Figures 2.2 and 2.3.

DEAE-Sephadex A50 column fractionation

Method 2. Flow-chart

Preparation of LD_1 from haemolysate:

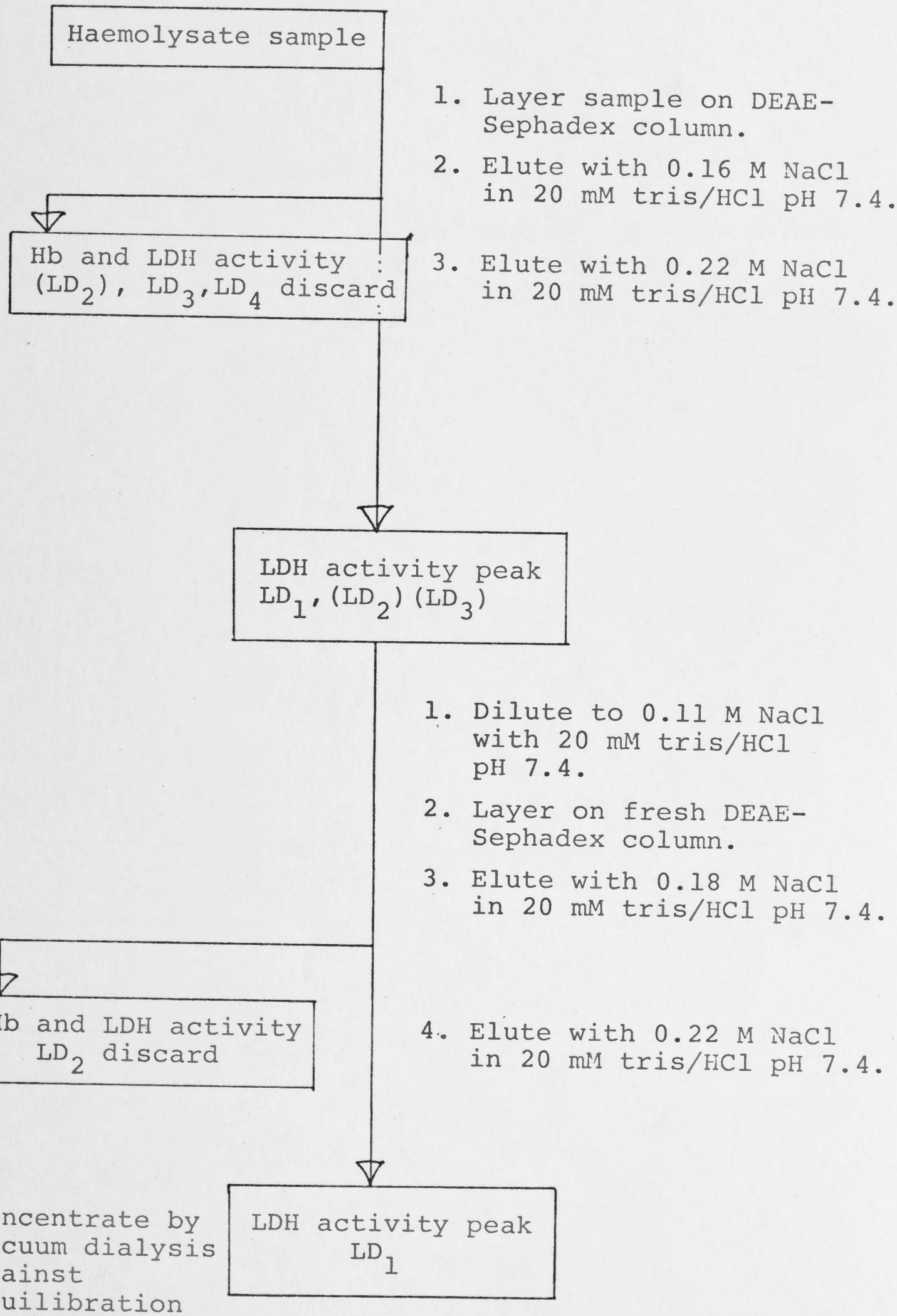


Figure 2.1 DEAE-Sephadex. Preliminary separation of the isoenzymes.

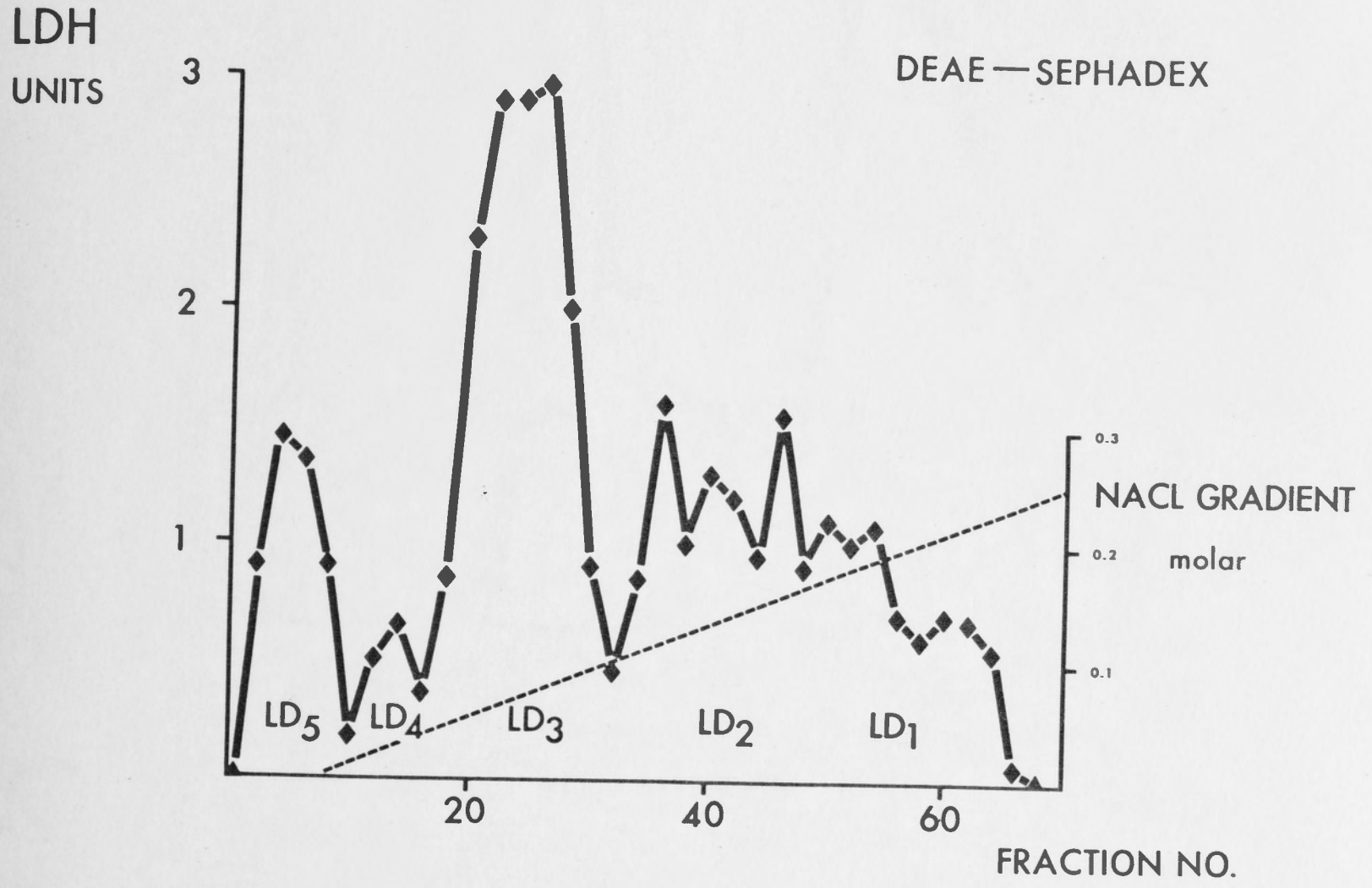
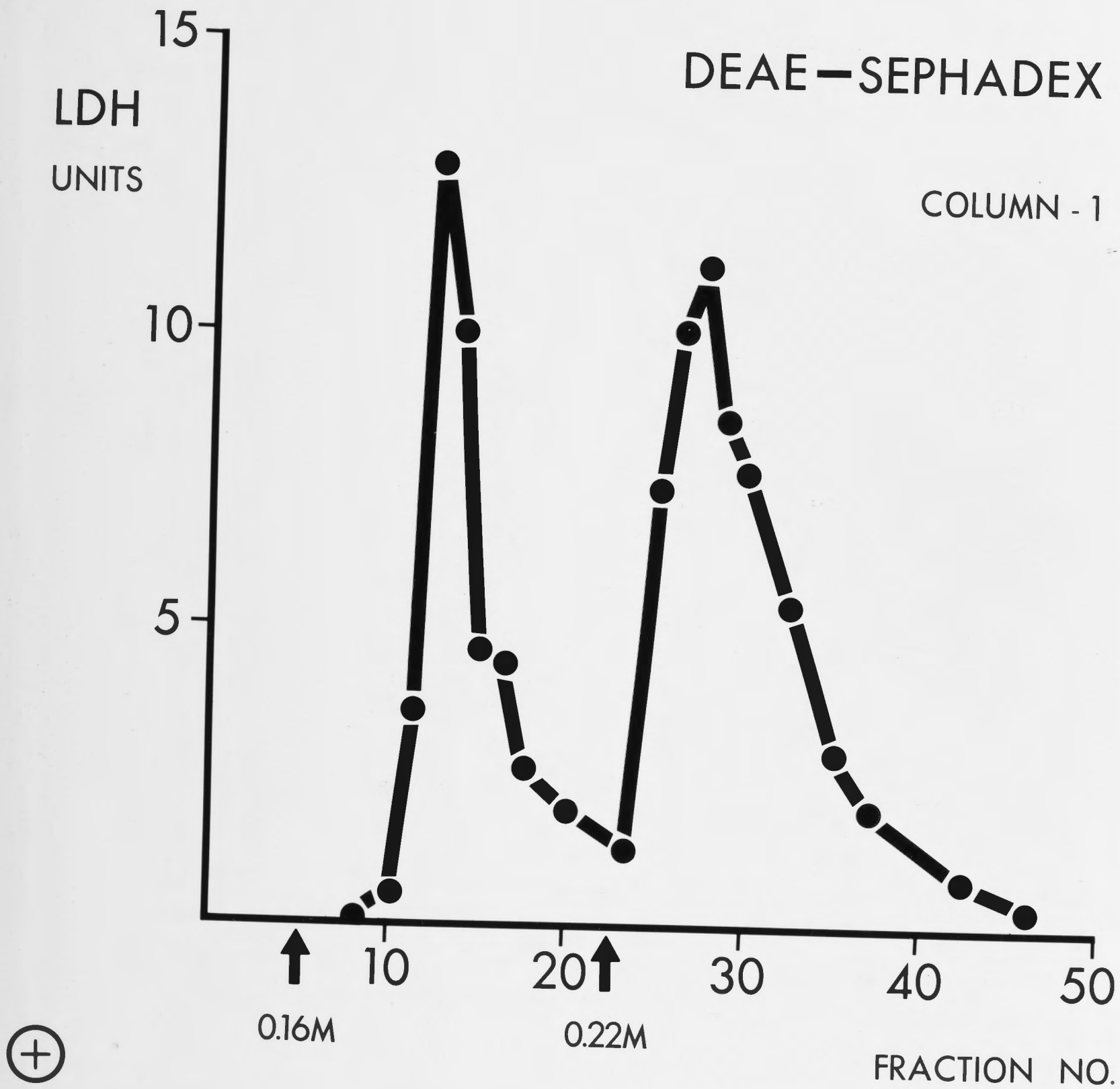

LDH
UNITS
3
2
1
DEAE — SEPHADEX
0.3
NACL GRADIENT
0.2
molar
0.1
LD5
LD4
LD3
LD2
LD1
20
40
60
FRACTION NO.

Figure 2.2 DEAE-Sephadex. Separation of LD_1 Column-1.


DEAE—SEPHADEX
COLUMN - 1
LDH
UNITS
15
10
5
10
20
30
40
50
0.16M
0.22M
FRACTION NO.

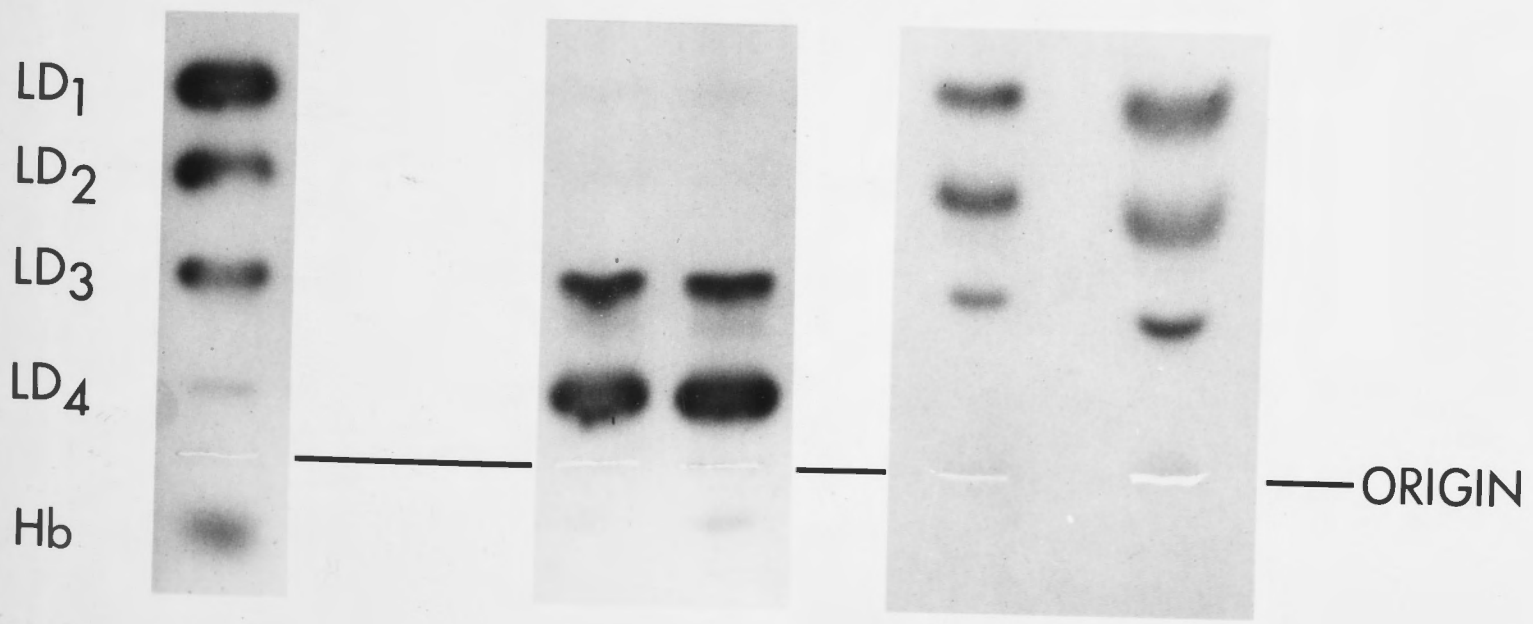
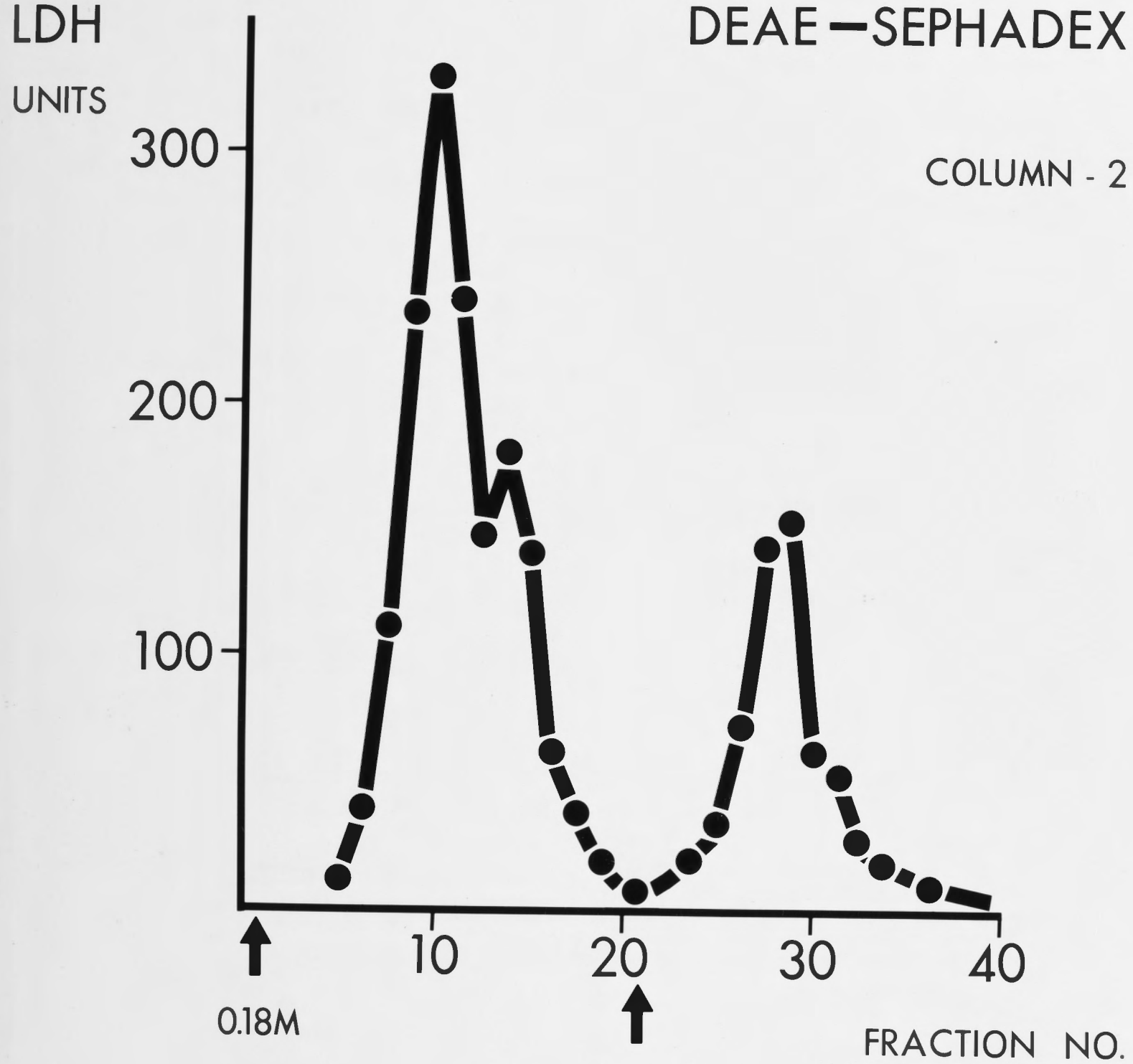
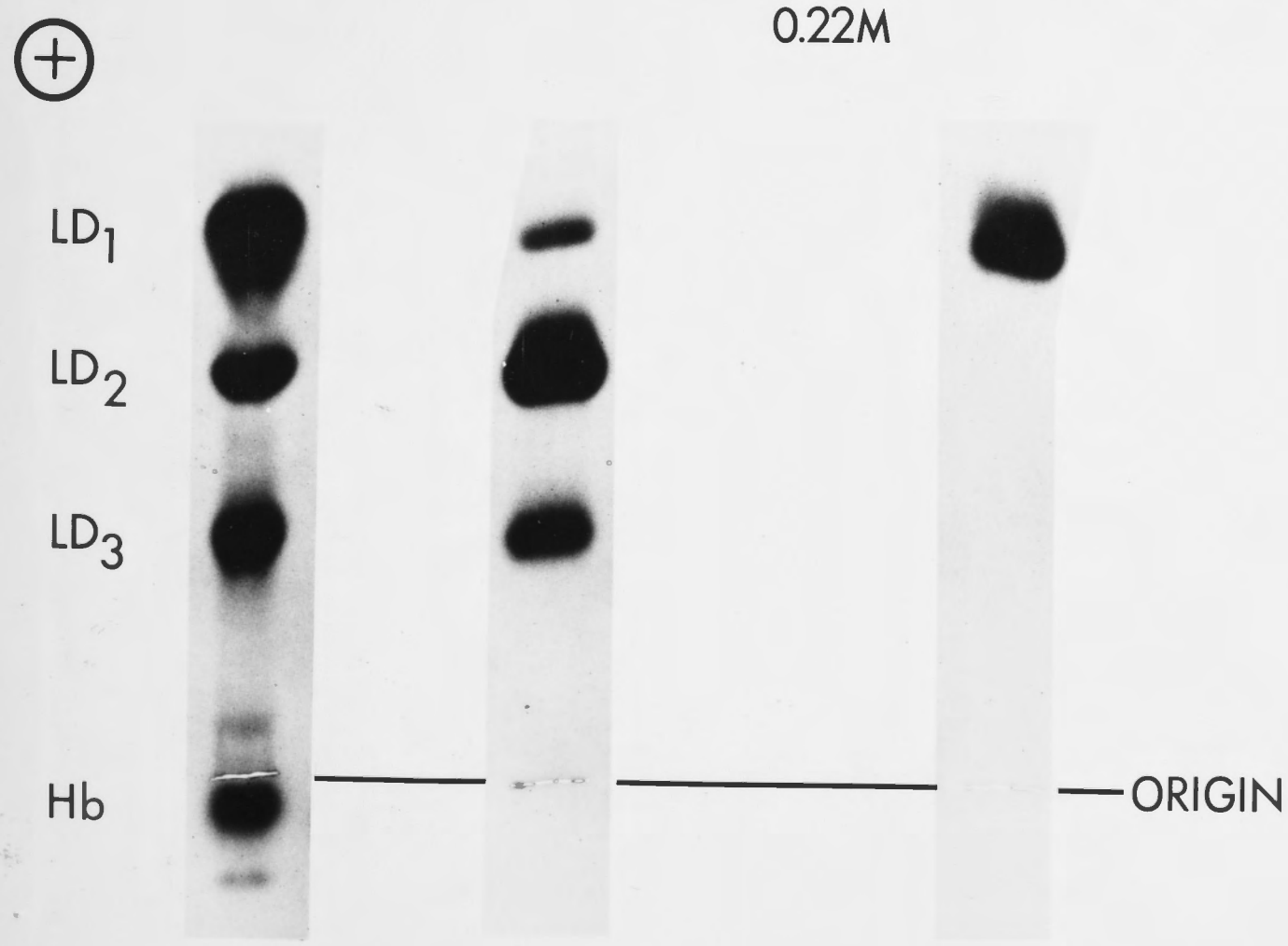

⊕
LD1
LD2
LD3
LD4
Hb
ORIGIN

Figure 2.3 DEAE-Sephadex. Separation of LD_1 Column-2.


LDH
UNITS
DEAE—SEPHADEX
COLUMN - 2
300
200
100
10
20
30
40
0.18M
0.22M
FRACTION NO.


⊕
LD1
LD2
LD3
Hb
ORIGIN

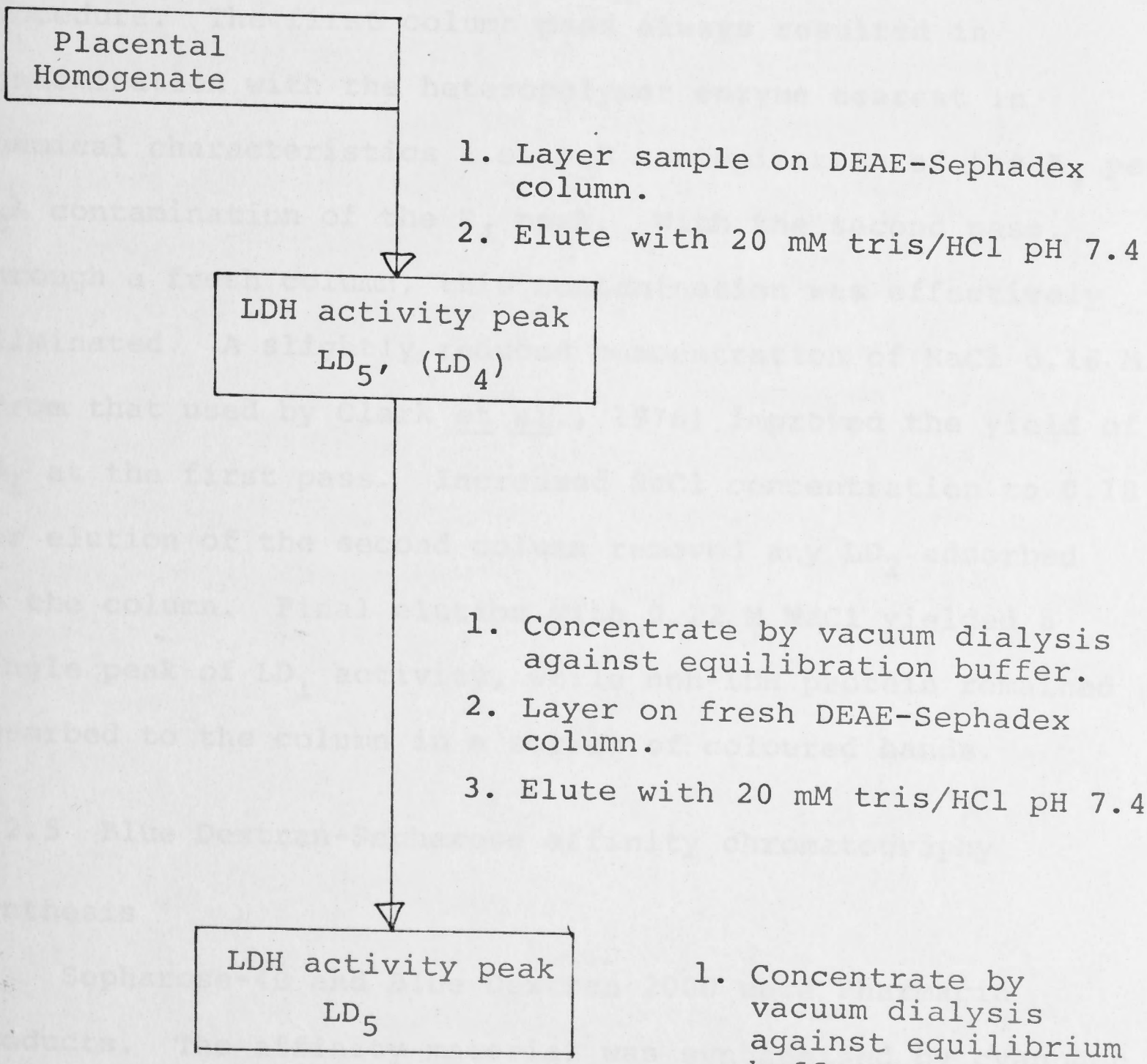
LD_5 preparation

Method 3. Following the layering of sample, equilibration buffer alone (3 column volumes) was passed through the resin bed. The resulting peak of activity was pooled, concentrated and after dialysis reapplied to a fresh DEAE-Sephadex column. The following flow-chart summarises the procedure.

DEAE-Sephadex A50 column fractionation

Method 3. Flow-chart

Preparation of LD_5 from placenta :



LDH activity coincident with a major peak of protein eluted following the void volume. This activity was LD_5 unrestrained by the DEAE, with some LD_4 as is seen in Figure 2.4. The majority of the chromogenic proteins were adsorbed in a broad coloured band at the top of the Sephadex bed. The second column yielded LD_5 free of other LDH activity - Figure 2.5.

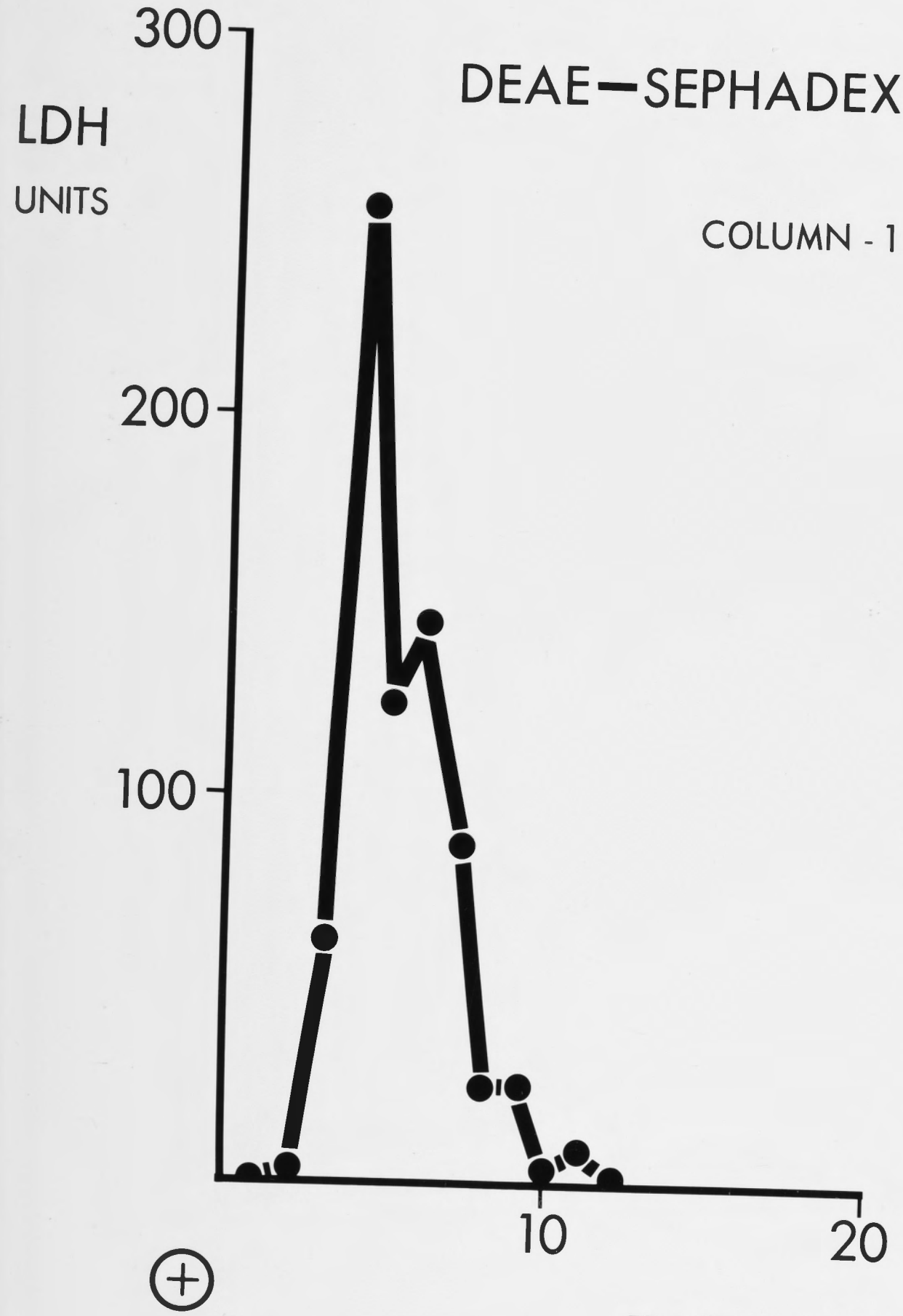
The DEAE-Sephadex pilot column, see Figure 2.1, provided useful information on the relative retention of the LDH isoenzymes by this material. Overlapping of the isoenzyme peaks occurred especially LD_2 with LD_1. A stepwise elution procedure was chosen to eliminate the protracted gradient procedure. The first column pass always resulted in contamination with the heteropolymer enzyme nearest in chemical characteristics i.e. A_3B contamination of the A_4 peak, B_3A contamination of the B_4 peak. With the second pass, through a fresh column, this contamination was effectively eliminated. A slightly reduced concentration of NaCl 0.16 M (from that used by Clark et al., 1976) improved the yield of LD_1 at the first pass. Increased NaCl concentration to 0.18 M for elution of the second column removed any LD_2 adsorbed to the column. Final elution with 0.22 M NaCl yielded a single peak of LD_1 activity, while non-LDH protein remained adsorbed to the column in a series of coloured bands.

2.2.5 Blue Dextran-Sepharose affinity chromatography

Synthesis

Sepharose-4B and Blue Dextran 2000 were Pharmacia products. The affinity material was synthesised by cyanogen

Figure 2.4 DEAE-Sephadex. Separation of LD_5 Column-1.


DEAE—SEPHADEX
LDH
UNITS
COLUMN - 1
300
200
100
10
20
FRACTION NO.

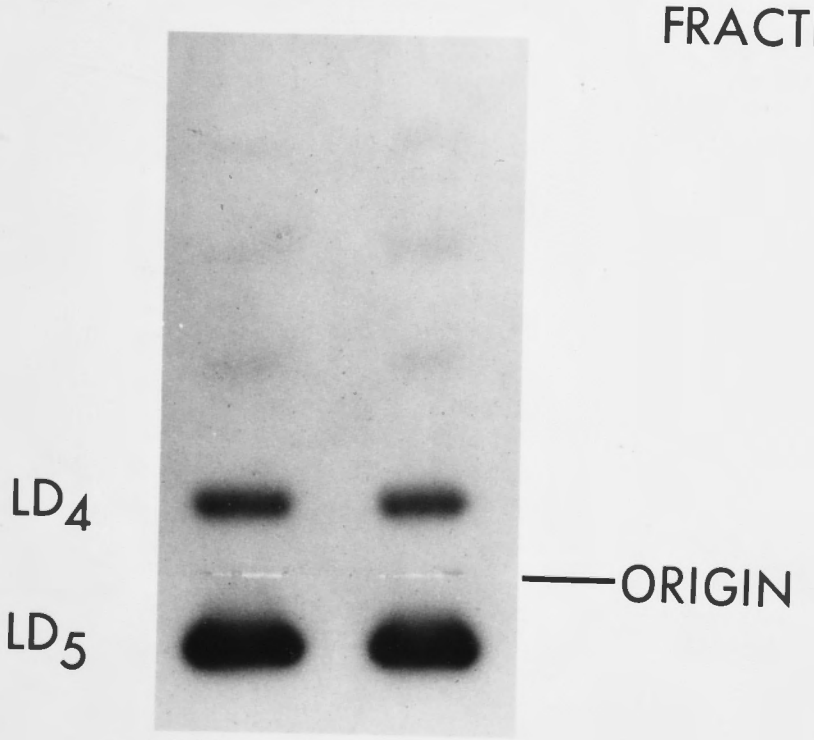
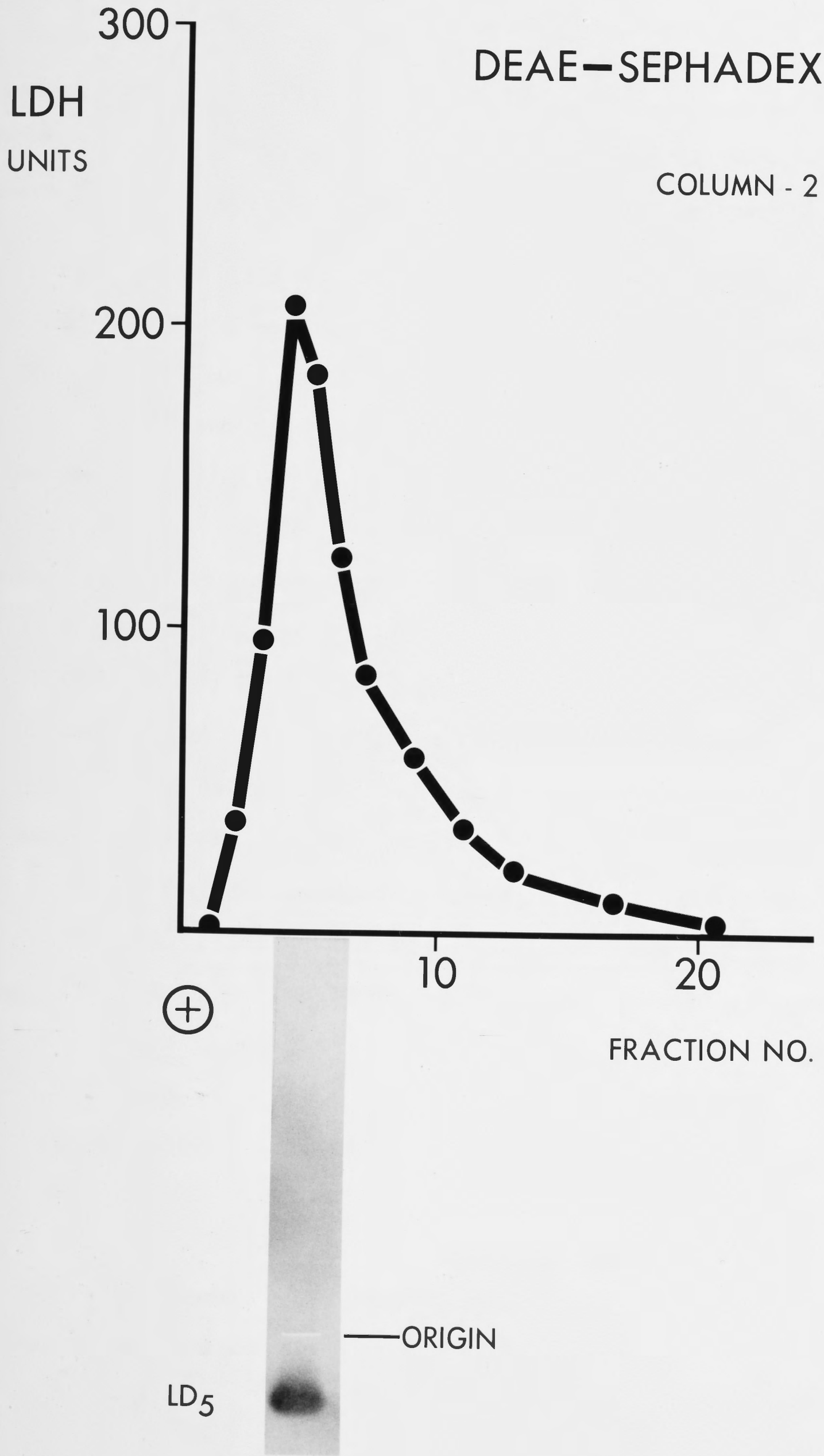

LD4
LD5
ORIGIN

Figure 2.5 DEAE-Sephadex. Separation of LD_5 Column-2.


DEAE—SEPHADEX
COLUMN - 2
LDH
UNITS
300
200
100
10
20
FRACTION NO.
⊕
ORIGIN
LD5

bromide activation of the Sepharose at pH 10±0.5 and the covalent bonding of Blue Dextran to this activated intermediate (Ryan and Vestling, 1974). The freshly synthesised material was thoroughly washed with 3 M KCl and by distilled water to remove all traces of unreacted CNBr and Blue Dextran.

LDH purification (after Nadal-Ginard and Markert, 1975).

The LDH preparation was dialysed against at least 4 changes, each of 6 hrs, of 20 mM phosphate buffer, pH 7.0, 1 mM in dithiothreitol, (DTT) at 4^{o}C and reduced in volume by vacuum dialysis if necessary. The sample was applied directly to the affinity column 2 cm × 30 cm which had been previously equilibrated with at least 10 column volumes of starting buffer.

Elution with starting buffer continued at 30 ml/hr until the protein concentration of the eluate fell to below 0.1 mg/ml. The column was then eluted with 2-3 column volumes of 1 mM NAD^{+} and 1 mM Li-lactate in 10 mM phosphate pH 7.0, 0.5 mM DTT, 2 column volumes of 10 mM phosphate pH 7.0, 0.5 mM DTT, and 2 column volumes of 0.5 mM NADH in 10 mM phosphate pH 7.0, 0.5 mM DTT. Finally the column was flushed with one column volume of 20 mM phosphate, 3 column volumes of 2.5 M KCl in 20 mM phosphate and 10 column volumes of 20 mM phosphate alone. Some LDH activity was found in the principal protein peak, which was unretarded by the column Figure 2.6. The amount of this activity was found to be related to the extent of sample dialysis and to the degree of total protein loading.

The equilibration buffer eluted the B_4 isoenzyme with traces of B_3A (Figure 2.6); the B_3A and B_2A_2 with some BA_3 were eluted by the pulse of NAD^+ and lactate, B_2A_2 predominating. Elution with NADH desorbed A_4 isoenzyme and the remaining BA_3 isoenzyme, when these isoenzymes were present in the sample as in the case of placental extract (Figure 2.7). When haemolysate was the sample, the NADH elution was omitted.

The final elution with KCl resulted in traces of all the heteropolymers (estimated at 3% of the applied activity) being eluted in a small protein peak.

An increase in specific activity up to 100 fold could be obtained in a single step from crude extracts of human tissues with this affinity material. At the same time a degree of differential fractionation of the isoenzyme was achieved. The bulk of the applied protein eluted in the void volume, unretarded and was conveniently monitored visually by the haemoglobin and/or myoglobin present. Exhaustive dialysis (6 changes of buffer) of the sample prior to application resulted in retention of the B_4 component (c.f. Figure 2.6 with Figure 2.7) which was then eluted with B_3A and B_2A isoenzymes by NAD^+ and lactate (Figure 2.7).

The B_4 isoenzyme has the strongest affinity for NAD, is therefore most sensitive to traces of endogenous NAD competing with the stationary ligand - coupled Blue Dextran. The differential binding of the B_4 and A_4 isoenzymes is

Figure 2.6 Blue Dextran-Sepharose. Haemolysate, fractionation of LDH isoenzymes.

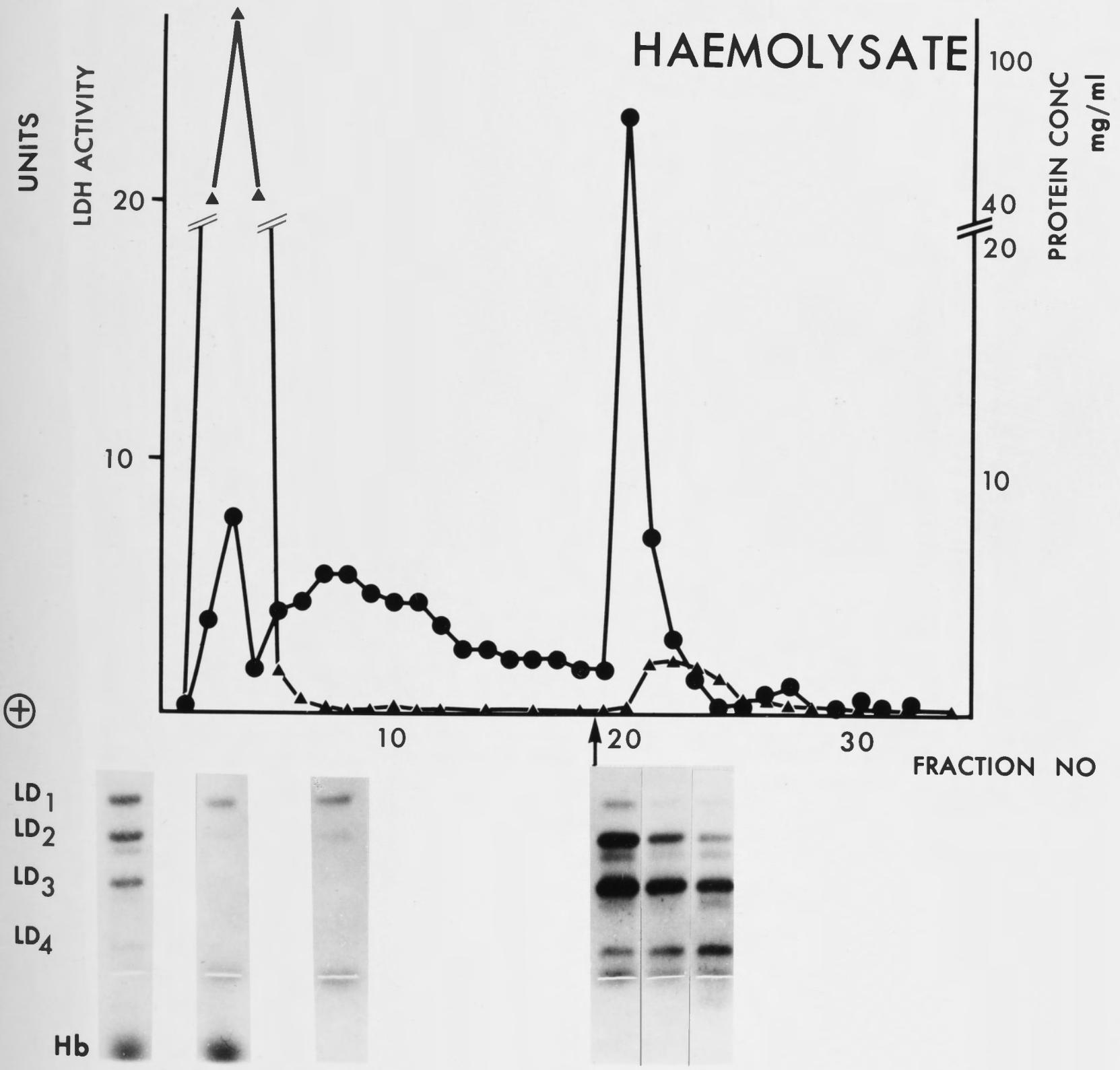
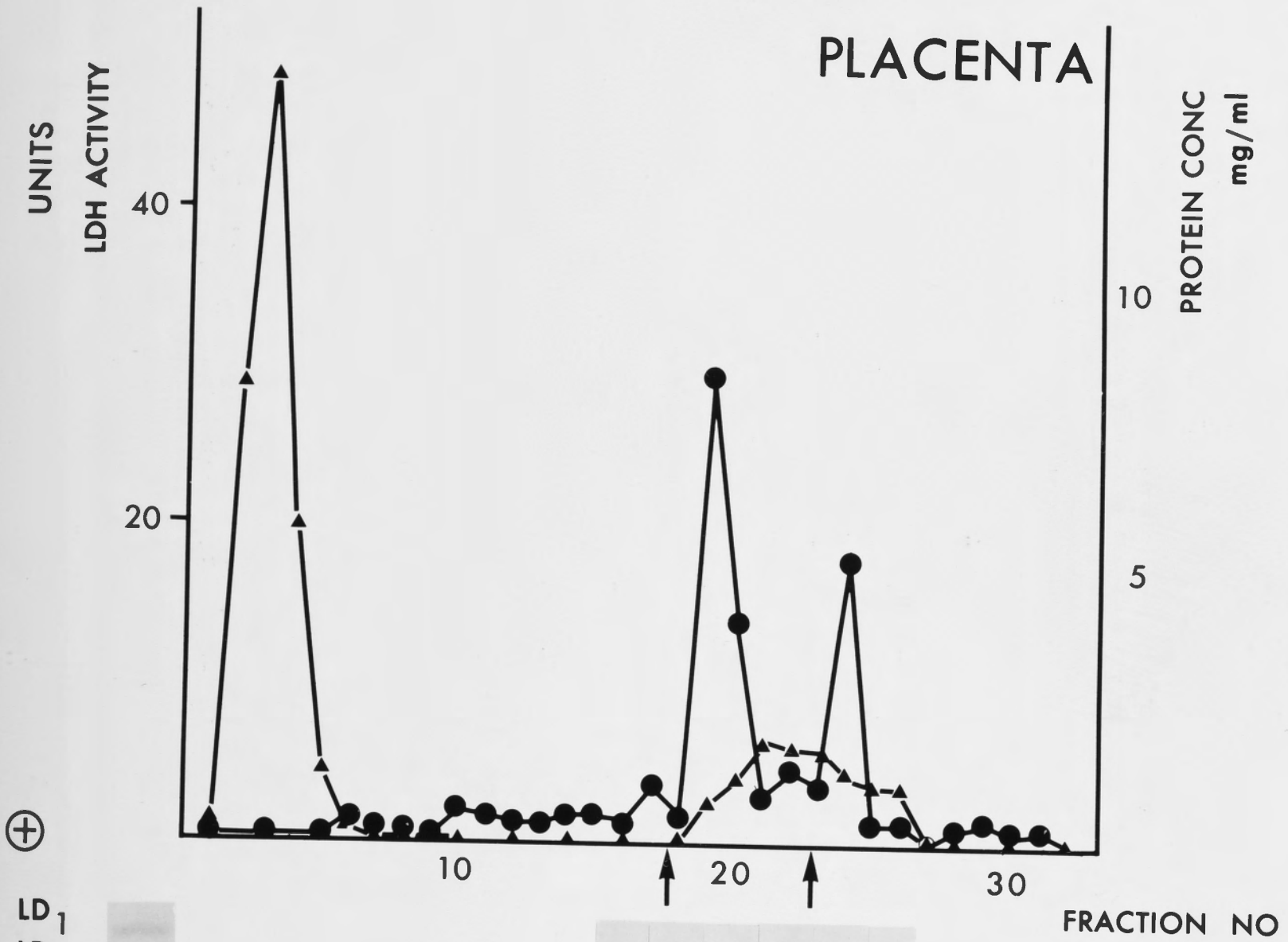

HAEMOLYSATE
UNITS
LDH ACTIVITY
20
10
100
40
20
10
PROTEIN CONC
mg/ml
10
20
30
FRACTION NO
LD1
LD2
LD3
LD4
Hb

Figure 2.7 Blue Dextran-Sepharose. Placenta, fractionation of LDH isoenzymes.


PLACENTA
UNITS
LDH ACTIVITY
40
20
10
PROTEIN CONC
mg/ml
10
5
10
20
30
FRACTION NO
⊕
LD 1
LD 2
LD 3
LD 4
LD 5

explained by this higher affinity of B_4 LDH for NAD and the adenine moeity in particular (Wieland, 1962). Blue Dextran is sufficiently similar, structurally, to NAD to be recognised by the dehydrogenase dinucleotide fold (Thompson et al., 1975). However the superior association constant of the biological nucleotide allows effective desorption of the enzyme by NAD present in the mobile phase. Affinity chromatography is a new and powerful biospecific tool in protein purification.

2.2.6 Placental DNA

An early sample of purified LD_1 from placenta was treated with glacial acetic acid to precipitate "core" material (as detailed in Chapter 7 - Peptide Mapping) prior to mapping. This precipitate possessed a low A_{280}/A_{260} ratio and following U.V. spectral analysis was considered to contain DNA. The peptide map resulting from the purification was poorly resolved and streaked in appearance. Although no reference to precautions against DNA contamination of LDH preparations was found in the literature, it was considered that DNA might be carried through the purification procedure to contaminate the final product.

Radio-labelled DNA tracer studies

Pure duplex viral DNA labelled with ^{32}P, 50,000 cpm was added to a LDH preparation from placenta and pancreatic DNAase-1 was added to a final concentration of 10 µg/ml, in a dialysis sac. Diffusion of the labelled oligonucleotide products of digestion into the dialysing buffer was monitored

by counting aliquots of the sac contents in the Perkin Elmer scintillation counter. The radioactivity of these sac aliquots declined over a period of 10 hours to a level approximately twice that of background activity.

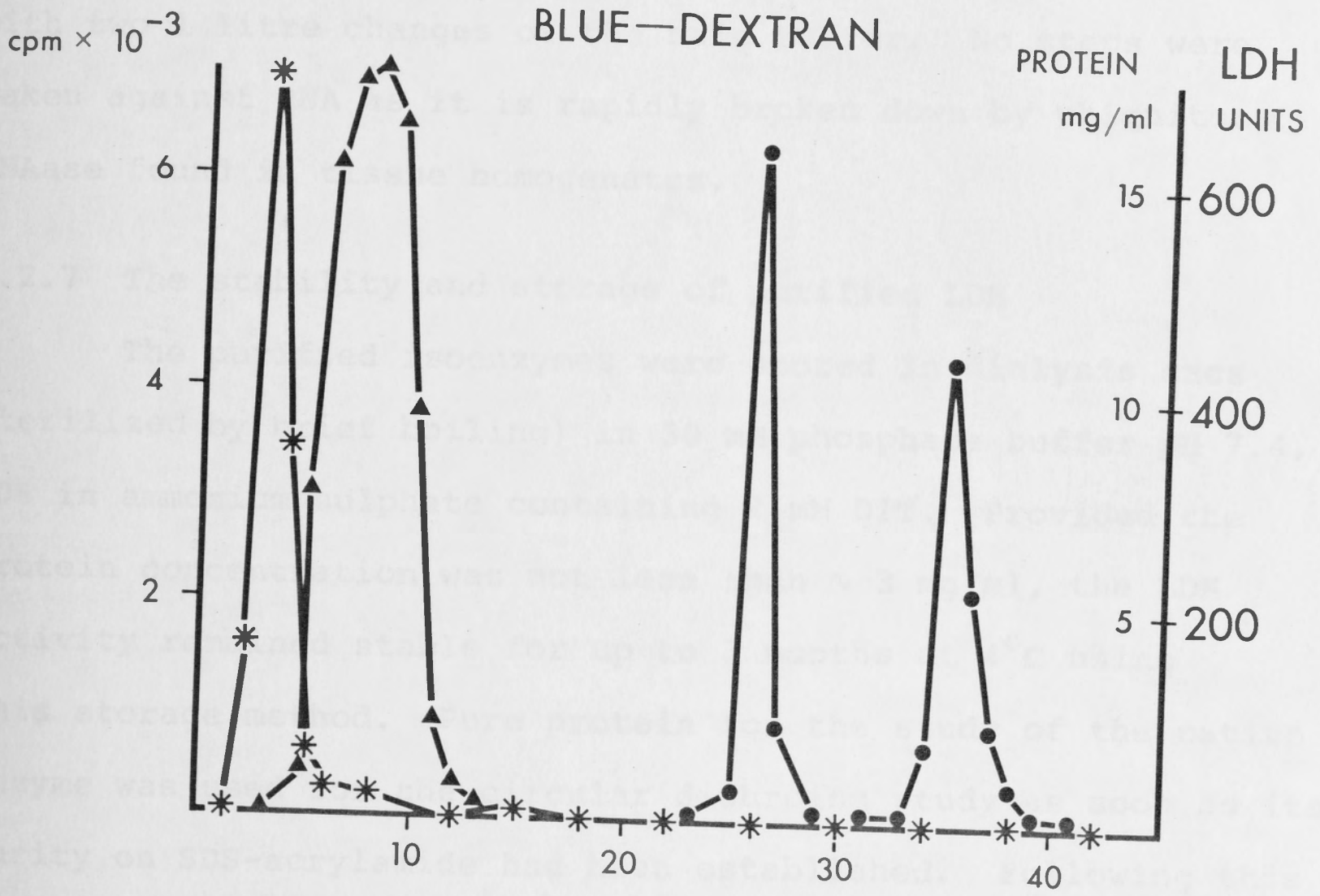
This pilot study demonstrated that DNA could be digested from a mixture of tissue homogenate and DNA by DNAase-1, while the LDH activity was not affected. To test whether undigested DNA was co-eluted with LDH, radio-labelled viral DNA was applied to a Blue-Dextran column mixed with a placental extract. Figure 2.8 demonstrates the rapid passage of this DNA through the column bed unretarded, in a major peak preceding the unretarded protein. Only radioactivity indistinguishable from background could be detected in the eluted LDH activity.

Although pure viral DNA was not retained on the Blue-Dextran column, human DNA, possibly in the form of nucleoprotein complexes possessing different physico-chemical conformation might be adsorbed to the affinity column. The recognition of nucleo-protein by a nucleotide analogue (Blue Dextran) and subsequent elution by an endogenous nucleotide (NAD in the mobile phase) is a possible explanation for DNA persisting in these preparations.

DNA digestion

Steps to eliminate the possibility of endogenous nucleic acid contamination of LDH purified from placenta were incorporated routinely into the purification following this study.

Figure 2.8 Radio-labelled DNA on Blue Dextran-Sepharose.



✳ RADIO LABELLED DNA

▲ PROTEIN

● LDH ACTIVITY

Crystalline pancreatic DNAase-1 (Worthington Biochemical) to 10 µg/ml was added to the partly purified LDH preparation after it had been dialysed against 20 mM tris-HCl pH 7.4 containing 10 mM $MgCl_2$ and 1 mM DTT. Digestion of endogenous DNA was allowed to proceed for 12-16 hours at room temperature with two 1 litre changes of the same buffer. No steps were taken against RNA as it is rapidly broken down by ubiquitous RNAase found in tissue homogenates.

2.2.7 The stability and storage of purified LDH

The purified isoenzymes were stored in dialysis sacs (sterilized by brief boiling) in 50 mM phosphate buffer pH 7.4, 70% in ammonium sulphate containing 2 mM DTT. Provided the protein concentration was not less than ∿ 3 mg/ml, the LDH activity remained stable for up to 3 months at 4°C using this storage method. Pure protein for the study of the native enzyme was used for the circular dichroism study as soon as its purity on SDS-acrylamide had been established. Following this study the same protein sample was freeze dried for the peptide mapping study and retained in this state under vacuum and dessication until required.

2.2.8 Summaries of purification

Summaries representative of these procedures are tabulated in Tables 2.1 and 2.2. DEAE-Sephadex chromatography precedes Blue Dextran in the purification of LD_1 from erythrocytes because of its superior property of eliminating haemoglobin and its products at an early stage in the purification. The DNA digestion step was not necessary with

haemolysates since red cells are known to possess very little DNA.

The homogeneity of the purified enzymes is demonstrated in Chapter 3.

Table 2.1 SUMMARY OF PURIFICATION OF ERYTHROCYTE LD_1

Purification step	Volume (ml)	Total protein (mg)	Total Activity (units)	Specific Activity**	Purification	Yield	Yield*
Haemolysate supernatant	1180+	12280	3170	0.258	-	100%	
DEAE-Sephadex 1st column	540	975	2250	2.62	10	80%	
DEAE-Sephadex 2nd column	550	346	1340	3.87	15	42%	
Blue-Dextran 1st column	32	51	998	19.60	76	32%	79%
Blue-Dextran 2nd column	10	4.8	280	58	226	9%	22%

+ Derived from 390 ml packed cell volume of erythrocytes.

* Yield of LD_1 assuming 40% of original activity is LD_1.

** Specific activity in units LDH activity per mg. protein.

Table 2.2 SUMMARY OF PURIFICATION OF PLACENTAL LD_5

Purification step	Volume (ml)	Total protein (mg)	Total Activity (units)	Specific Activity**	Purification	Yield	Yield*
Homogenate supernatant	344+	4860	2520	0.519	-	100%	
Ammonium sulphate 35-36%	420	1218	2020	1.66	3.2	80%	
Blue-Dextran 1st column	25	174.3	1850	10.61	20	73%	
Blue-Dextran 2nd column	15.5	46.5	1407	30.3	58	56%	
DEAE-Sephadex 1st column	27	12.0	1238	103.2	199	49%	
DNAase treatment							
DEAE-Sephadex 2nd column	5.6	2.49	519	208	401	21%	82%

\+ Derived from 113 g wet weight of placenta

* Yield of LD_5 assuming 25% (Wieme, 1959) of original activity is LD_5

** Specific activity in units LDH activity per mg. protein.

3. ELECTROPHORETIC INVESTIGATION OF CALCUTTA-1 LDH

The Calcutta-1 variant was first recognized as an electrophoretic variant differing from the normal LDH isoenzyme bands on starch gel in that fast anodal bands were present. The investigation of the electrophoretic variants of other proteins (Chapter 1) suggests the altered migration of the variant was often explained by an amino-acid substitution. A fast anodal migration implies the addition of nett negative charge which in terms of substitutions suggests either a neutral to acidic substitution or a basic to neutral substitution. A range of electrophoretic techniques were therefore considered appropriate to probe the nature of the Calcutta mutation.

3.1 Factors affecting the electrophoresis of proteins

The nett charge developed by a protein depends on the sum of the component acidic and basic amino acid residues and the extent of their ionization, which is largely determined by the pH of the electrophoresis buffer. The nett charge and hence the mobilities of proteins are greater at buffer pH values removed from their isoelectric point.

The isoelectric point of a particular protein is defined as that pH at which the sum of its dissociated acidic groups is equal to the sum of its dissociated basic groups. Arginine, pK 12.5 and lysine, pK 10.3 (determined in the free amino acids) possess relatively strong basic side groups while aspartic acid, pK 3.7 and glutamic acid, pH 4.3 are relatively strongly acidic. Several other amino acids possess weakly

acidic or basic side chains e.g. histidine, pK 6.0; cysteine, pK 8.3 (acidic) and tyrosine, pK 9.8 (acidic). The isoelectric point for the whole protein is principally determined by the algebraic sum of the arginines, lysines, aspartic and glutamic acid residues. In addition, these four residues are quantitatively more common components of many soluble proteins. The effective pK of any particular ionizable amino acid side chain will be influenced by its location in the protein structure and in particular by its environment of neighbouring amino acid residues. Any particular ~~such~~ residue will therefore exhibit a unique effective pK. Fisher et al. (1977) have determined the isoelectric points of human LD_1 and LD_5 by isoelectric focussing techniques as 9.20 ± 0.09 from 9 determinations for LD_5 and 4.55, range 4.50-4.58 from 3 determinations for LD_1. These values can be attributed to the greater lysine plus arginine content of LD_5 than LD_1 as well as the greater aspartate plus glutamate content of LD_1 than LD_5.

Constraints on the practical demonstration of enzymes such as LDH by specific enzyme staining after electrophoresis in gel supporting-media are imposed by the pH dependence of the reaction catalysed by the enzyme (see Chapter 5) as well as by the degree of enzyme stability at extremes of pH. In support media such as starch and acrylamide gels, sieving of the protein molecules occurs, dependent on the protein size in relation to pore size of the gel, and is a factor of importance in comparative studies of proteins.

3.2 Electrophoretic techniques

A number of techniques of starch gel and acrylamide gel electrophoresis were utilized for particular purposes in the investigation of the Calcutta-1 variant. These experiments included the study of the native enzyme, the study of the purified protein subunits and the determination of protein purity of the enzyme preparation.

3.2.1 Study of the native enzyme by starch gel electrophoresis

Routine visualization of LDH isoenzymes during purification was made by electrophoresis on horizontal starch gel slabs 19 cm × 16 cm × 0.5 cm. The principal buffer system used was that of Blake and Kirk (1969a), 0.2M sodium phosphate/citric acid pH 7.0, see Appendix-1. Gels were made 12½% w/v in starch and samples were applied on Whatman 3MM paper wicks. Electrophoresis proceeded at 5 volts/cm for 16 hrs or in some cases for 4 hr at 4-6^{o}C in the coldroom. Gels were sliced and the isoenzyme bands developed by the tetrazolium staining method of Fine and Costello (1963).

Double length gels 38 cm × 16 cm × 0.5 cm were used to enhance separation of the variant bands with the time of running increased to 24-40 hours. Alternative buffer systems used are tabulated in Appendix-1. Placental extracts electrophoresed on 12½% w/v starch gels using the buffer systems 1-4 tabulated in Appendix-1, characteristically demonstrated the presence of all five isoenzymes of LDH - Figure 3.1 a,b,c. Only two bands were observed at LD_4 when variant placental extract was subjected to electrophoresis at pH 7.0, see Figure 3.1.d. These bands are clearly

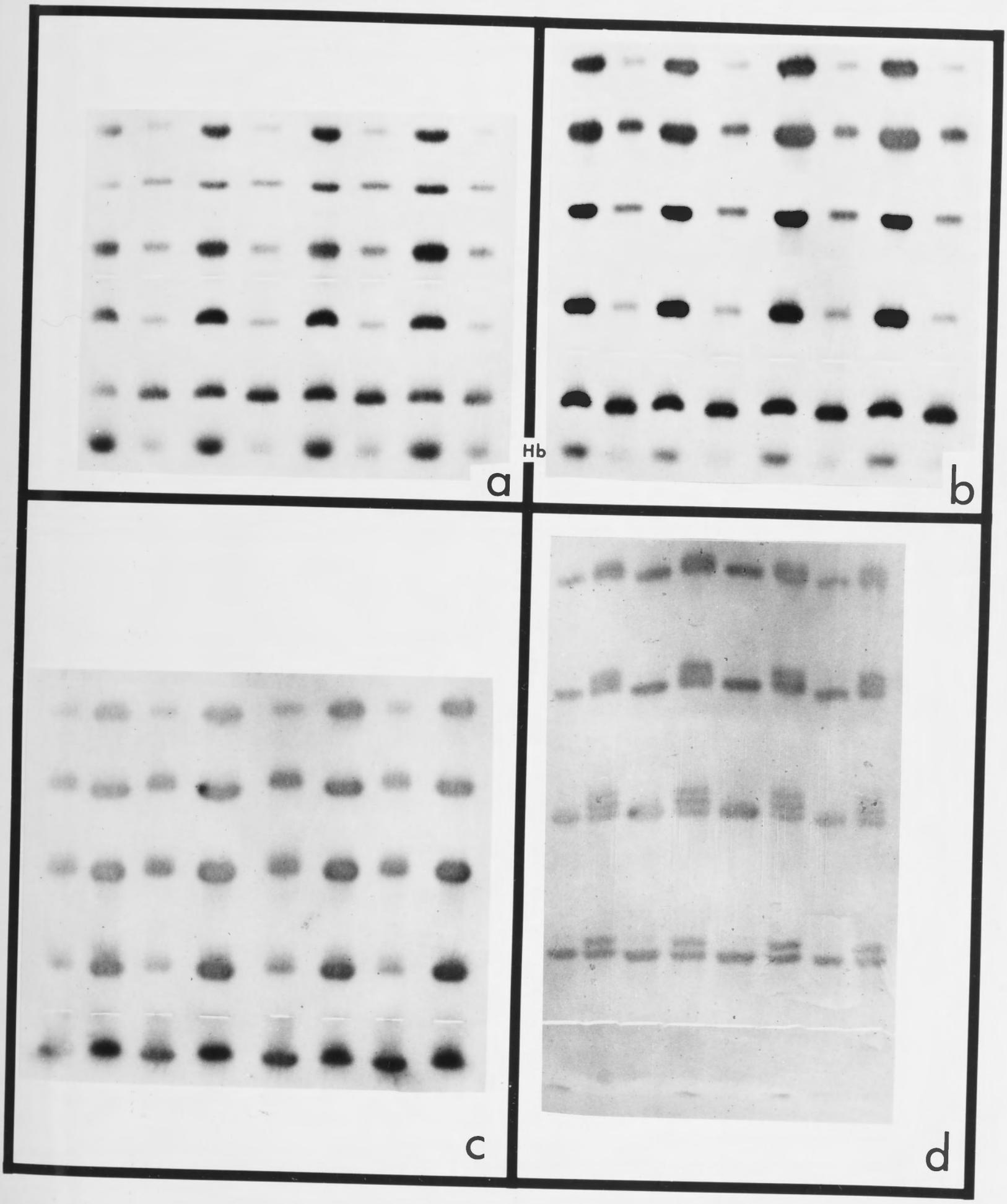
Figure 3.1 Placental samples stained for enzyme, anode at the top. Sample concentrations balanced for LD_5 activity

a. Starch gel pH 5.0 citrate-phosphate, alternately from the left, normal, Calcutta. Origin slots central, between LD_3 and LD_4.

b. Starch gel pH 6.0 citrate-phosphate, alternately normal, Calcutta. Origin between LD_4 and LD_5.

c. Starch gel pH 7.0 citrate phosphate, alternately Calcutta, normal. Origin between LD_4 and LD_5.

d. Double-length starch gel pH 7.0, citrate-phosphate, alternately normal, Calcutta. Origin cathodal to LD_4.


Hb
a
b
c
d

separated and appear to be of equal intensity, the slower band aligning with the normal BA_3 band, while the other is anodal. This observation is consistent with a B mutation, the bands being interpreted as BA_3 and βA_3. Using buffers of differing composition and ionic strength at pH 8.0 e.g. buffer 4 of Appendix 1, an anodal component of variant LD_4 is observed - see Figure 3.2 a,b,c, but the component of normal migration seen on the double length pH 7.0 gel - Figure 3.1 d, is not obvious.

In all buffer systems tried, Calcutta LD_5 appeared as a <u>single</u> band, sometimes with <u>fast</u> anodal migration relative to the normal LD_5 - see Figures 3.2 a,c, sometimes with <u>slow</u> anodal migration relative to normal LD_5 - see Figures 3.1 a,c, and particularly in Figure 3.1 b; sometimes with the <u>same</u> migration as normal LD_5, - see Figure 3.2 b. Calcutta LD_5 never presented an appearance of multiple bands under any of the electrophoretic conditions tested.

Anodal components of LD_1, LD_2 and LD_3 were best demonstrated in the double length gel systems - see Figures 3.1 d and 3.2 a. The broad LD_1 region of the heterozygote variant could not be resolved into bands on a starch gel, even when the sample was pretreated with up to 5 mM 2ME prior to application to the gel. This pretreatment had been found in the Canberra laboratory to sharpen the bands of LDH activity in some haemolysates. Moreover the anodal migration of

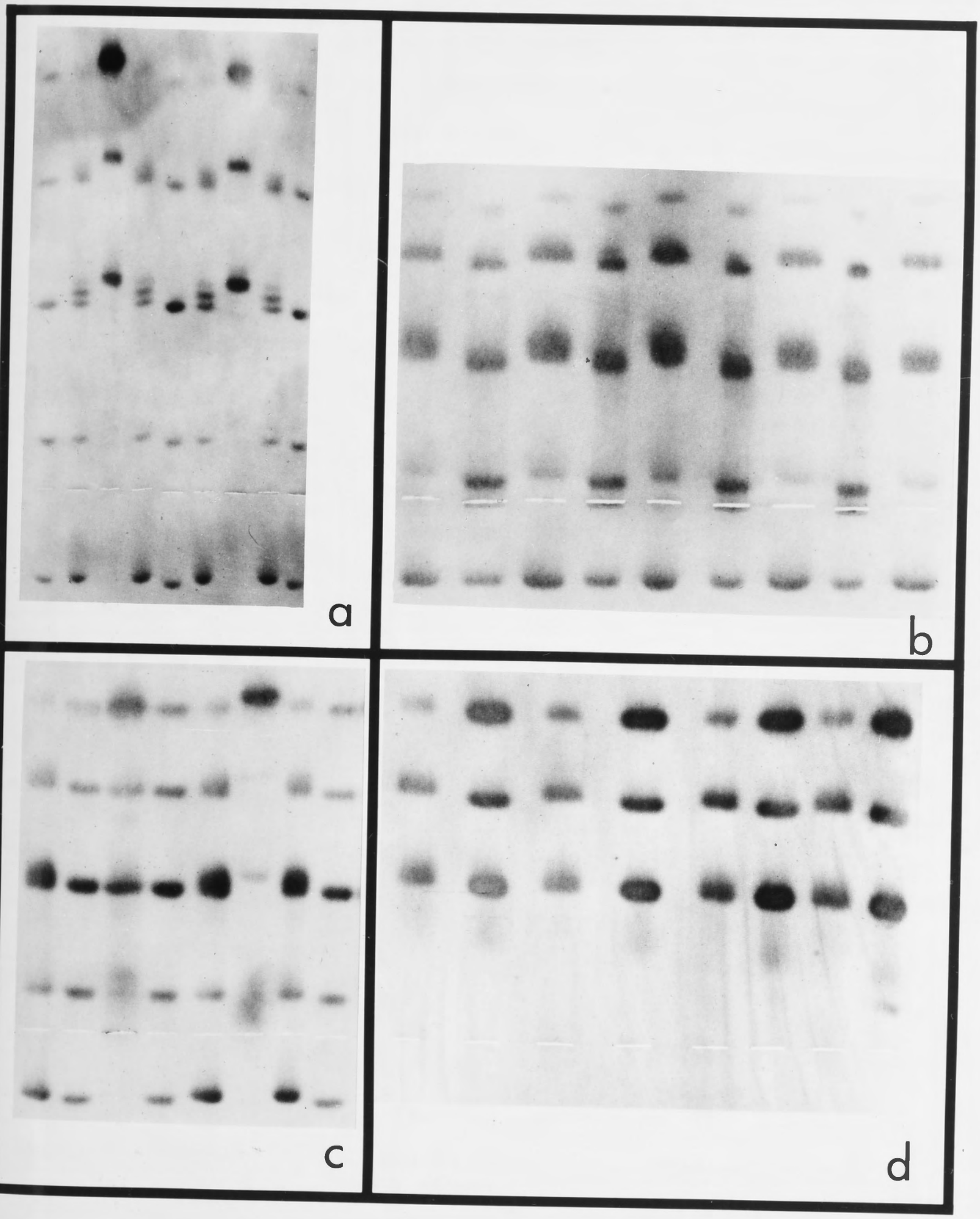
Figure 3.2 Placental and erythrocyte samples stained for enzyme, anode at the top. Origins between LD_4 and LD_5

a. Double length starch gel pH 8.0, tris-phosphate 0.2 M, samples from the left, normal placental, heterozygote Calcutta placental, homozygote Calcutta erythrocyte, heterozygote Calcutta placental, normal placental, etc.

b. Starch gel pH 8.0, tris-phosphate 0.1 M, samples alternately Calcutta, normal.

c. Starch gel pH 8.0, tris-phosphate 0.2 M, samples heterozygote Calcutta, normal placental, normal erythrocyte, normal placental, heterozygote placental, homozygote Calcutta,etc.

d. Starch gel pH 8.0, tris-HCl 0.2 M, samples alternately Calcutta heterozygote placental, normal placental.


a
b
c
d

Calcutta LD_1 relative to normal LD_1 was always demonstrable throughout all stages of protein purification and in all the buffer compositions and pH's used. These procedures involved exposure of the isoenzymes to high ionic strength salt solutions, changing buffer composition, repeated dialysis and protein concentration and dilution. These processes should have removed loosely attached molecules which might have affected the electrophoretic migration of the enzyme.

The variability of the migration of Calcutta LD_5 relative to normal LD_5 depending on buffer composition, pH and time of electrophoresis, compared with the consistent anodal migration of the Calcutta LD_1 no matter what buffer system was used, suggested that an anodal mutation was more likely to have affected the B subunit than the A subunit. The differences in Calcutta LD_5 migration might be artifacts of the electrophoresis conditions.

3.2.2 Study of the native enzyme by polyacrylamide gel electrophoresis

The electrophoresis of crude Calcutta-1 haemolysates on polyacrylamide disc gels results in an LDH isoenzyme banding pattern indistinguishable from that of normal haemolysates. Even when haemolysates shown to be heterozygous or homozygous for Calcutta by starch gel electrophoresis, were run on disc acrylamides by colleagues in the Canberra laboratory, the variants appeared the same as normals.

However when purified Calcutta-1 heterozygote LD_1 was electrophoresed in highly sieving disc acrylamide gels, separation of the anodically fast bands of the variant was achieved. This resul_ in a 11% gel is shown in Figure 3.5 a.1 with three bands of Calcutta LD_1 activity visualized by enzyme stain. The slowest band corresponds to normal LD_1 which in similar gels runs as a single band, and the two faster bands are variant components. In an 8% disc gel (Figure 3.5 a.2) the three bands of activity show enhanced separation with close to equal inter-band spacing. Band 1 corresponds to normal LD_1 while bands 2 and 3 are variant bands. The spacing between bands 1 and 2 is the same as the spacing between bands 2 and 3. A similar relationship is seen between the three bands in the 11% gel using the same Calcutta preparation. Band 3 carries one more unit of charge than band 2 and band 2 carries one more unit of charge than band 1. Figure 3.5 b illustrates the homogeneity of the Calcutta LD_1 and normal LD_1 preparations on SDS-disc gels - Buffer system Appendix 2. This close comparability between the migration of LD_1 Calcutta and LD_1 normal on SDS-acrylamide confirms a very similar, nearly identical molecular weight of the variant and normal subunits and the faster mobility of bands 2 and 3 in Figure 3.5 a must be due to increasedcharge carried by the variant.

To further develop the thesis that the Calcutta mutation might be explained by a charge mutation in the polypeptide chain, alkaline urea dissociation electrophoresis was carried out.

3.2.3 Study of the purified protein subunits

The purified proteins were reduced in mercaptoethanol and dissociated in 7M urea according to the method of McKenzie and Treacy (1973). After allowing overnight dissociation at 4°C, the A subunits from the purified LD_5's were subjected to acid urea starch gel electrophoresis in formate/NaOH pH 3.0, 7M urea (Bell, 1966). Electrophoresis proceeded for 7 hours at room temperature and the gel was then removed, fixed and stained with nigrosine. Figure 3.3 shows the result of this experiment. Rabbit muscle LD_5 (Sigma), further purified on DEAE-Sephadex to remove traces of contaminant proteins, was included as a control. Migration of the A subunits of Calcutta heterozygote are seen to be the same as that of the normal A subunits.

The B subunits from the purified LD_1's were separated in pH 8.8 tris/glycine, 7M urea (McKenzie and Treacy, 1973) on 11% acrylamide gels. An earlier attempt using the same buffer/urea system in starch gel produced streaming bands. Figure 3.4 shows the results obtained after fixing and staining for protein. A single principal band was observed for normal LD_1. In the equivalent position, two bands appear in the Calcutta sample, showing better separation at the lower protein loading. The last 3 gels in Figure 3.4 show the same enzyme preparations after passage through a Sephadex G-200 mini-column (as the native enzyme) to remove the persistent lower molecular weight contaminant. The depletion of the faster β band is apparent. One possible explanation for this may be lowered stability of the

Figure 3.3 LD_5 subunit migration on starch gel pH 3.0 7M in urea. N = normal LD_5, RM = rabbit muscle LD_5, C = Calcutta heterozygote LD_5.

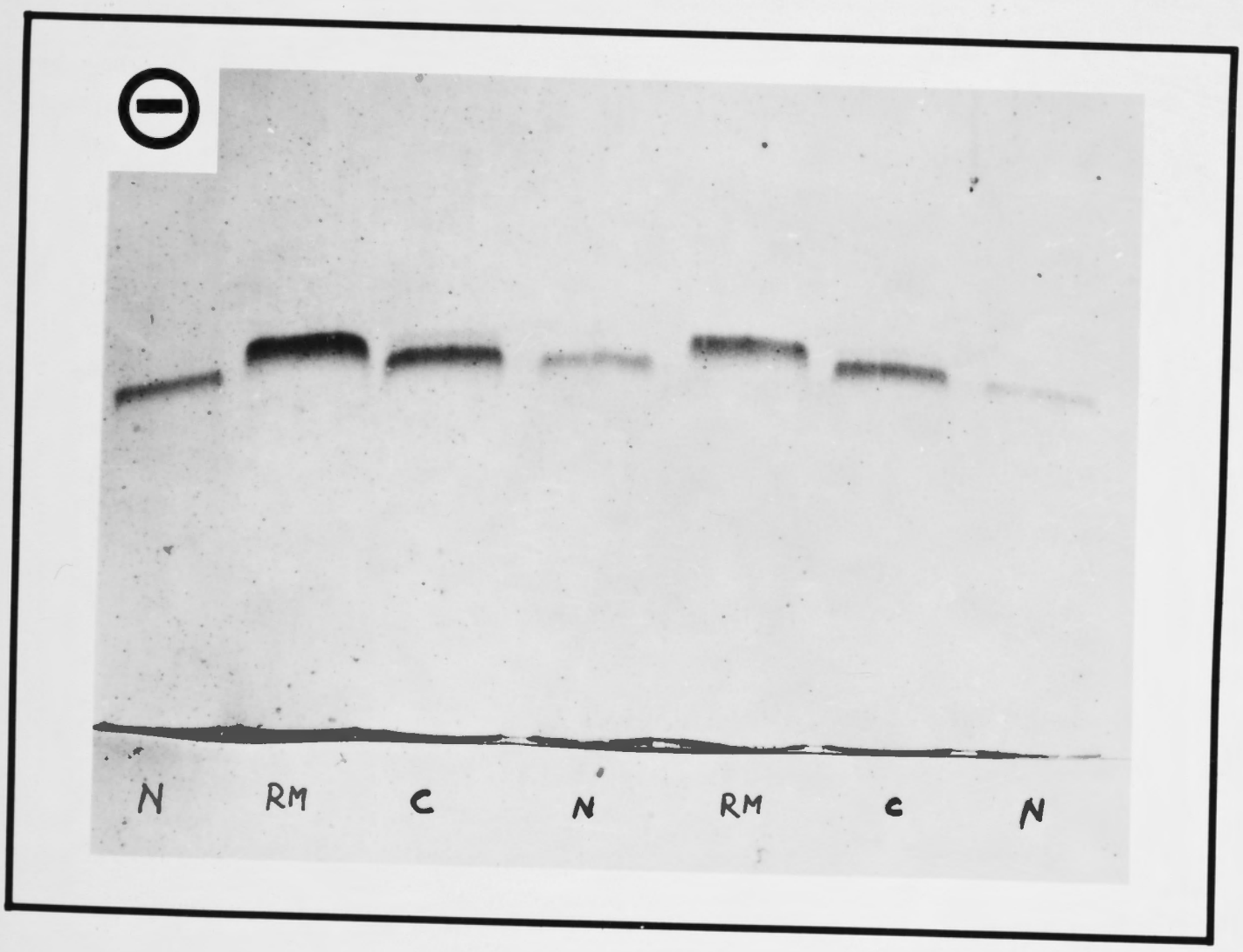

N
RM
C
N
RM
C
N

Figure 3.4 LD_1 subunit migration on 11% acrylamide disc gels pH 8.8 in 7M urea. Coomassie blue protein stain. N = normal LD_1, C = Calcutta het. LD_1.

From LHS: The first gel n shows a single principal band of normal LD_1 and a weaker fast contaminant band.

In the next gel c the Calcutta LD_1 (marked by two dots) separates as two bands of equal intensity. The fast contaminant band is also present.

The third gel depicts the same Calcutta preparation at lower sample loading. The two principal bands are clearly visible.

The group of three gels at the right of the figure show samples of the same enzyme preparations which have been passed through a Sephadex G-200 mini-column, prior to urea dissociation, to remove the fast minor contaminant band seen on the three previous gels. Band 1 is the single subunit B of normal LD_1. The Calcutta sample shows two bands marked by dots. One is of equivalent migration to band 1 of normal and a further, faster band 2 which is the β subunit. See text for an explanation of the relative depletion of band 2.

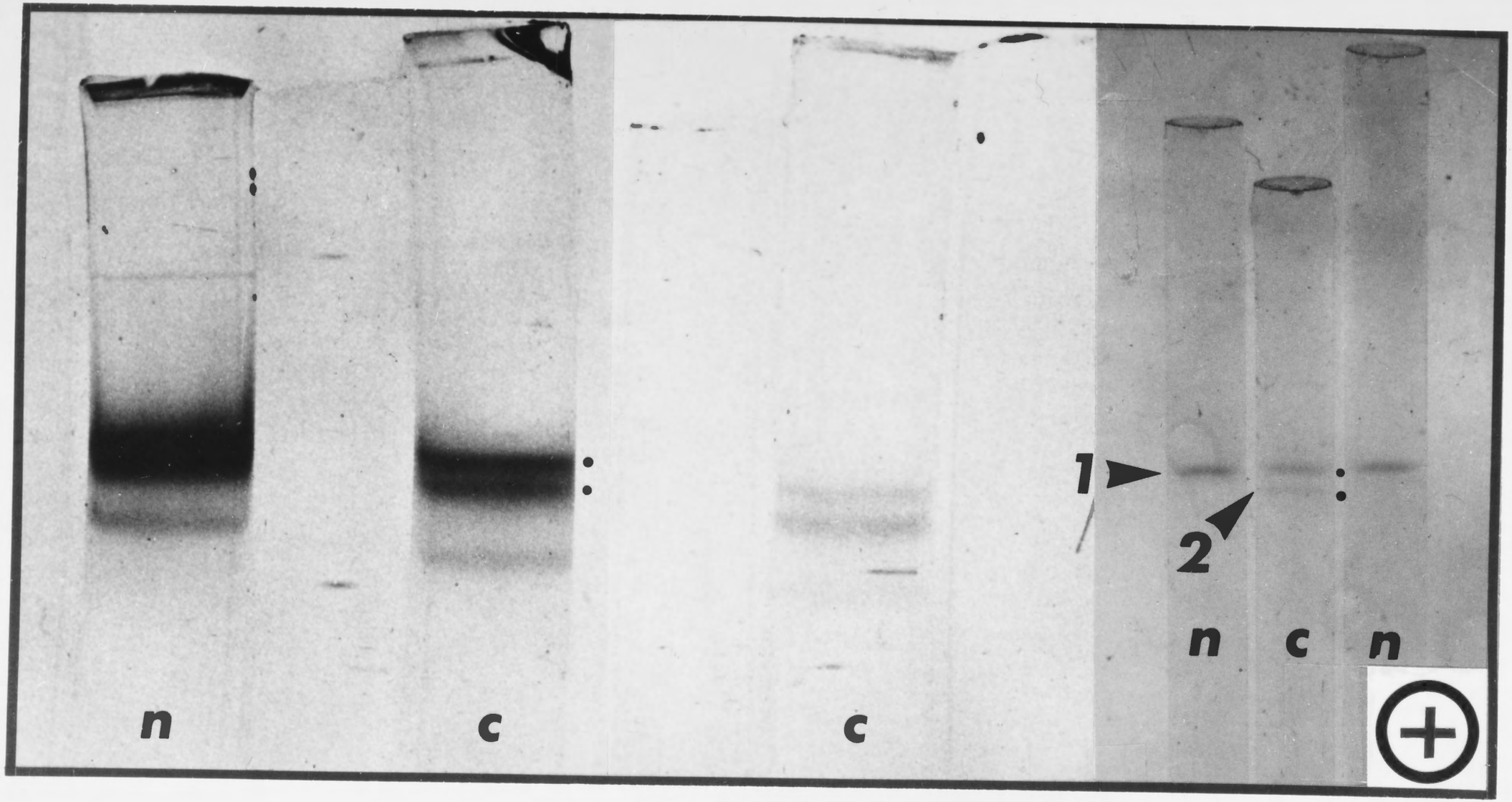
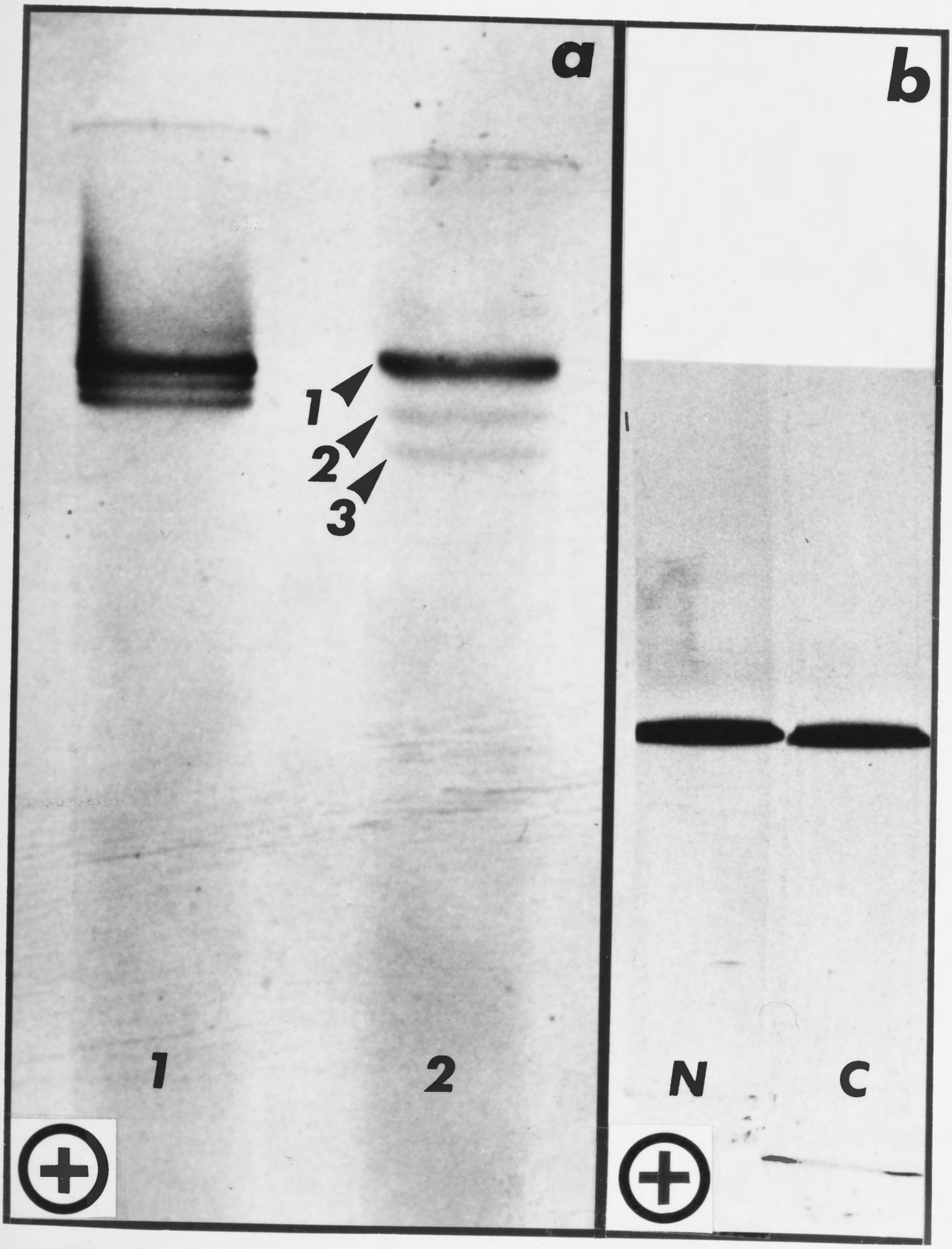

n
c
c
1
2
n
c
n

Figure 3.5 Polyacrylamide disc gel electrophoresis of purified LD_1's.

a.1 Calcutta-1 heterozygote purified LD_1, enzyme stained. 11% acrylamide disc gel, origin at the top, buffer system Appendix 2 No.2.

a.2 The same sample. 8% acrylamide disc gel, other details as for a.1. Band 1 corresponds to Normal LD_1 (B_4) Band 2 is a fast variant component probably $B_3\beta$, and band 3 is a further fast variant component probably $B_2\beta_2$.

b. SDS-acrylamide disc gel 5%. Buffer system Appendix 2 No.1. N = normal LD_1 C = Calcutta-1 heterozygote LD_1 (same sample as in (a) above.


a
b
1
2
3
1
2
N
C

β-rich LD_1 tetramers $B\beta_3$ and β_4. If these components tended to be labile at the late stages of purification when protein concentration on the mini-column was low then the β band would be under-represented among the polypeptide chains following dissociation.

3.2.4 Isoelectric focusing of native enzyme in thin-layer acrylamide gels.

The broadening of the LD_1 band from Calcutta-1 heterozygote seen on the starch gel (Figure 3.1 d) suggested that several unresolved bands might be present. Analytical electrofocusing in polyacrylamide gels is a powerful method for resolving proteins of differing isoelectric point. Access to the equipment required for this technique recently became available to the author and has enabled the purified normal and Calcutta LD_1 enzymes to be compared.

LKB precast polyacrylamide gel plates containing Ampholines covering the ranges pH 3.5-9.5 and pH 4-6.5 were used in conjunction with the LKB 2117 Multiphor system connected to a 2000 V constant wattage power supply. Purified enzymically active enzyme solutions were concentrated 10-fold in an Amicon 8 MC cell fitted with the microvolume accessory under nitrogen pressure. Samples of 15 ul containing 20-50 ug protein were applied to the gel surface using Whatman 3 MM paper wicks. Trial runs with beef heart LD_1 were made to optimise the sample loading, the loading position in relation to the electrodes, and the focusing time. Electrofocusing under constant power with

pumped cooling water at 10°C was completed in 2-4 hours. The slabs were stained for protein using Coomassie Brilliant Blue R250 by staining protocol 1 in the LKB Application Note 250 (December, 1977).

The results show that both beef LD_1 and normal human LD_1 focus as single major bands. Minor bands are also present. Calcutta-1 LD_1 focused as a series of closely spaced bands. At least five Calcutta bands (Figure 3.6 a) are apparent in the pH 3.5-9.5 gel, the anodically slowest band 2 aligning with the normal LD_1 band 1 of adjacent tracks. The second and third fastest bands show increasing amounts of protein, (best observed in Figure 3.6 b, third Calcutta track from the left). The higher non-focused background precludes assessment of the relative amounts of protein in the fourth and fifth fastest bands. The observed result is consistent with the expected pattern of intensity discussed in Section 1.5, at least in the first three bands of the Calcutta samples ....

B_4	$B_3\beta$	$B_2\beta_2$	$B\beta_3$	β_4
1	4	6	?(4)	?(1)

The close range pH 4-6.5 gel, Figure 3.7, presents an electrofocusing picture much more difficult to interpret than the wide range gel. It was hoped that the pI values of each of the Calcutta bands would be ascertained from this gel. At the anodic end of the banding pattern, Calcutta and normal LD_1's share common sharply focused bands (numbers 4 and 5 in Figure 3.7 b). The beef LD_1

Figure 3.6 Isoelectric focusing of purified LD_1's.

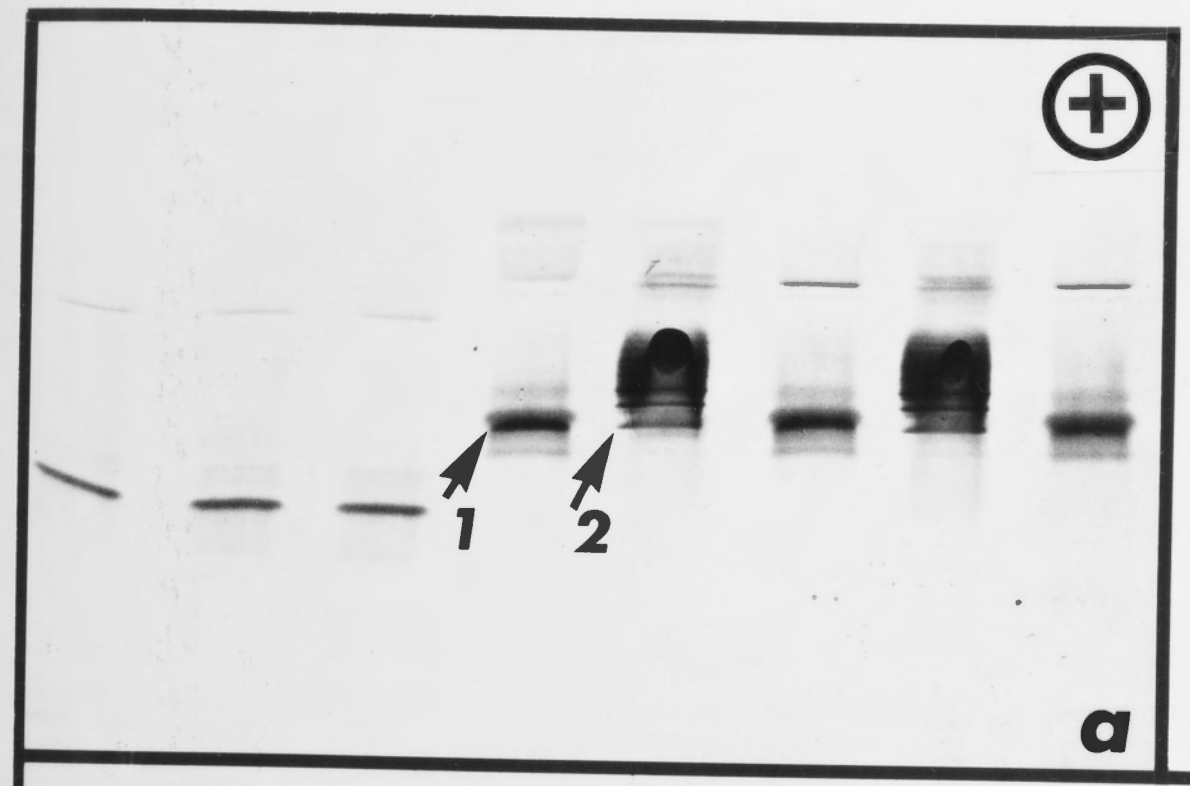
a. Beef 20 ug loading, normal human LD_1 20 ug, Calcutta 30 ug. Note band 1 of normal LD_1 exactly aligns with band 2 of Calcutta LD_1.

Samples from LHS: 3 tracks of Beef LD_1, normal human LD_1, Calcutta het. LD_1, normal, Calcutta, normal.

b. Repeat experiment of a. above, using same samples except that normal LD_1 was diluted to give a 10 ug load. Calcutta was not diluted. Note the loss of sharpness and increased anodal mobility of the normal sample.

Samples from LHS: 3 tracks of Beef LD_1, normal human LD_1, Calcutta het. LD_1, normal, Calcutta, normal, Calcutta, 2 tracks of mixed normal and Calcutta.

IEF details. PAG plate pH 3.5-9.5, Total acrylamide concentration(T) = 5% crosslinker concentration(C) = 3%. Ampholine = 2.4%, anode buffer 1M H_3PO_4, cathode buffer 1M NaOH. Limiting power = 7 watts, potential at focus (4 hr). 500V, stain Coomassie Blue R250.


1
2
a

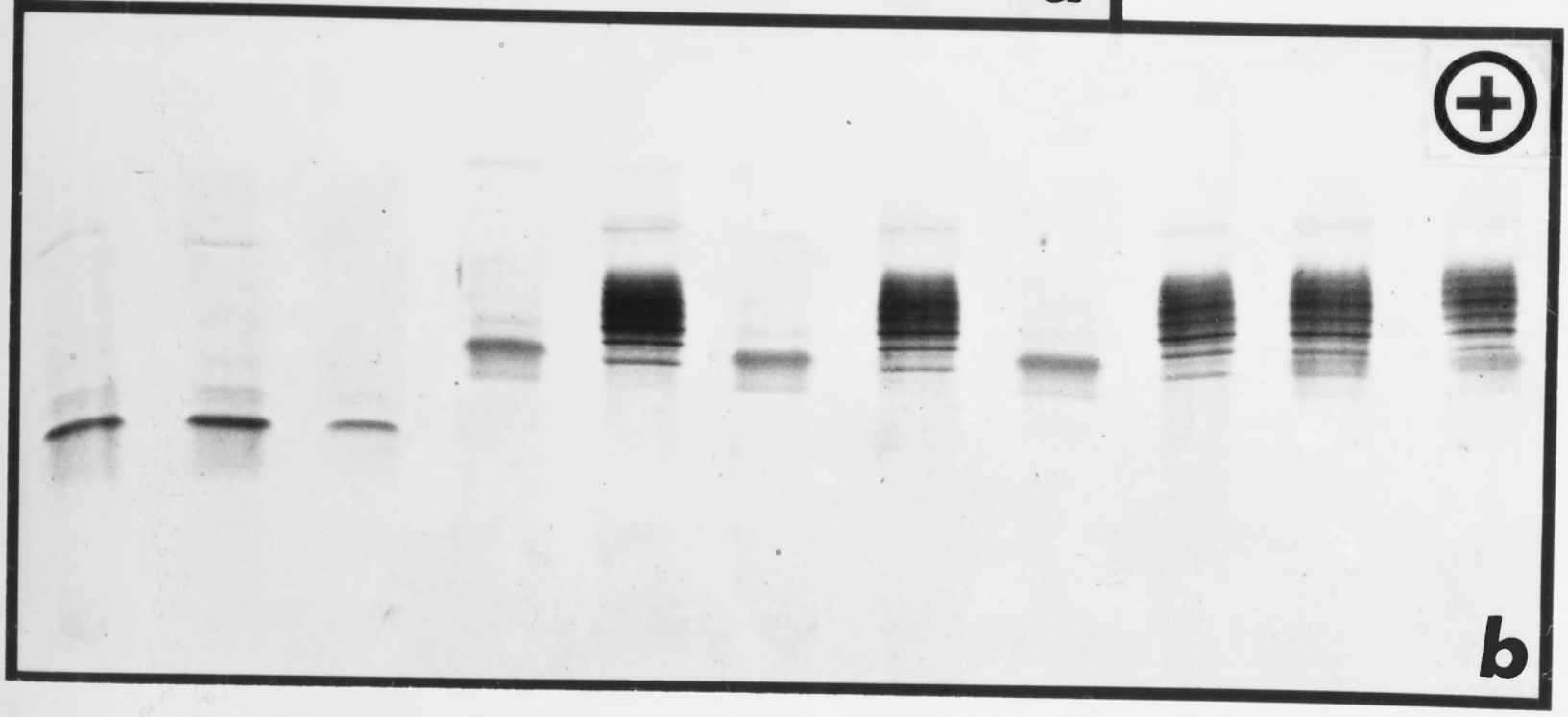
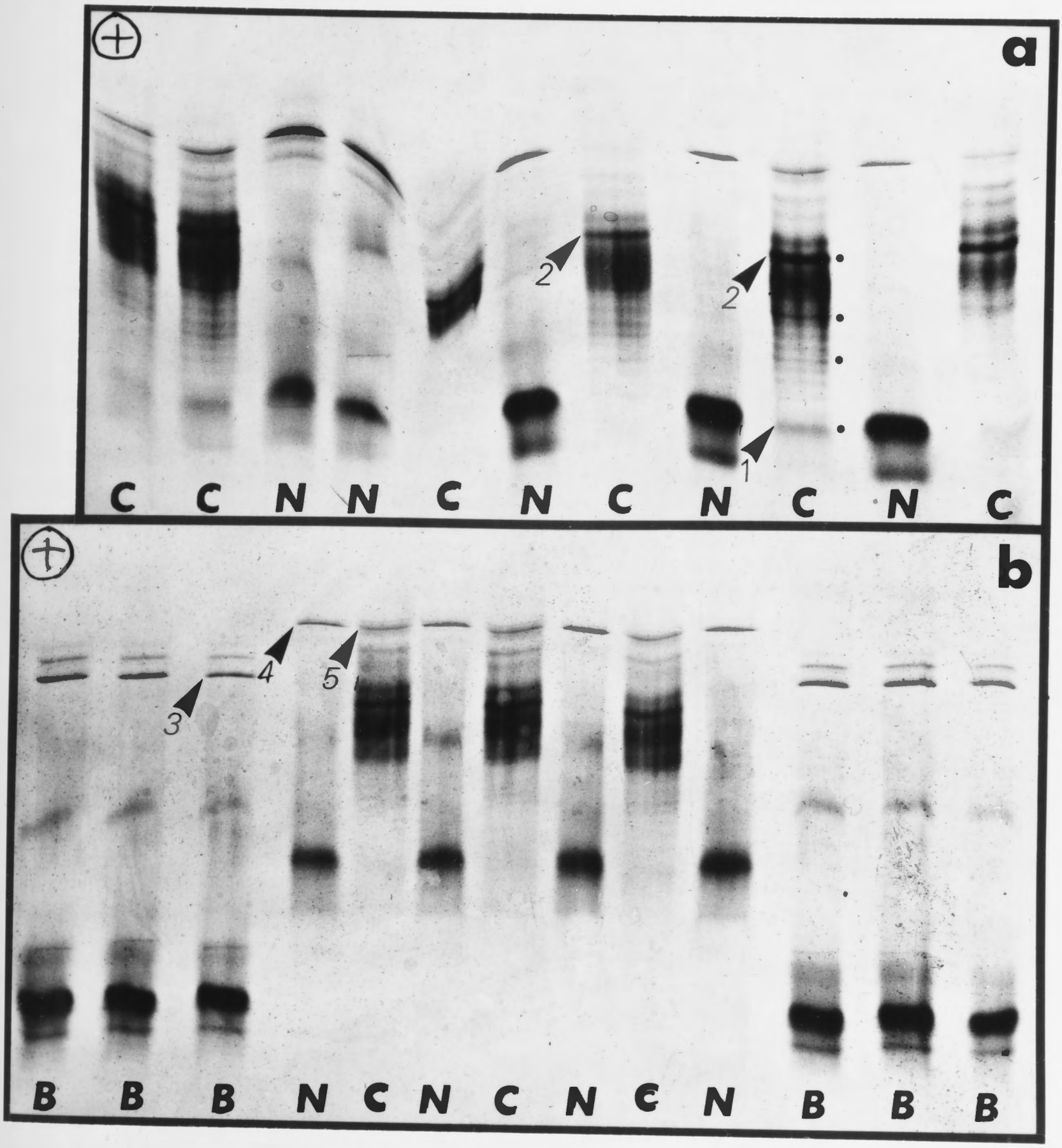

b

Figure 3.7 Isoelectric focusing of purified LD_1's on close range pH gel 4 - 6.5

a. N=normal, C=Calcutta, Normal 20 ug loading, Calcutta 30 ug loading. Dots mark Calcutta bands whose pI values are listed in Table 3.1. Band number 1 of Calcutta is the equivalent of the principal band of normal LD_1 adjacent. Band 2 is the strongest of the Calcutta bands.

b. Repeat of experiment a. above, using the same samples, with a Beef LD_1 control. B=Beef LD_1 30 ug loading. Bands 3,4,&5 may be acidic degradation products of LD_1's.

IEF details. PAG plate pH 4 - 6.5, T=5%, C=3%, Ampholine= 2.4%, anode buffer 1M H_3PO_4, cathode buffer is 2% Ampholine 5-7. Limiting power=10 watts, potential at focus (2.5hr.) 1280V, stain Coomassie blue R250.


a
C C N N C N C N C N C
2
2
1
b
3
4
5
B B B N C N C N C N B B B

control demonstrates three such bands in the equivalent position, the slowest marked number 3 (Figure 3.7 b). These minor bands may represent a modification or degradation product of the focusing process. Calcutta LD_1 demonstrates a major band (marked number 2) in a region of heavy staining with minor bands from the position of the main band of normal (number 1) through to the sharp "front" band (number 5). At least 16 Calcutta bands can be seen (Figure 3.7.a dotted track). Some of these bands may be the possible monomeric, dimeric, trimeric as well as tetrameric combinations of a normal B subunit and a mutated β subunit i.e. B (Band 1), β, Bβ, B_2, β_2, $B_2\beta$, $B\beta_2$, B_3, β_3, $B_3\beta$, $B\beta_3$, $B_2\beta_2$, B_4, β_4. Fourteen bands could be accounted for in this way, but modification products may also be present.

The problem of a high degree of heterogeneity on isoelectric focusing of proteins shown to be homogeneous by the commonly accepted criteria, is not a new phenomenon. Chamoles et al. (1970b) showed human LD_5 to focus in multiple bands with pI's between pH 8 and 9. Thorstensson et al. (1975) showed that human heart LDH (including isoenzymes LD_1-LD_4) focused into three groups of bands. Three families of bands at pH 5.0-5.9, 6.1-6.8, and 8.5-9.0, totalling more than five bands of focused heart LDH were noted by Dale and Latner (1968) in agreement with the results of Chamoles and Karcher (1970b). Weller et al. (1973) note that even crystalline, high specific activity rabbit muscle LD_5 demonstrated a major and two minor components on electrofocusing and suggest modification or degradation as the cause.

Measurement of isoelectric points.

The pH gradient generated by the focused ampholines was measured by the careful use of a special surface micro-electrode. pH readings at one centimeter intervals were plotted on the graph provided by LKB. When the fixed, stained PAG plate was overlayed, the intersection of each protein band with the plotted gradient gave a direct measure of pI. The results obtained appear in Table 3.1.

Table 3.1

PAG plate	Enzyme	Band	pI.	Chamoles et al. (1970a)	Thorst-ensson et al. (1975)	Fisher et al. (1977)
3.5-9.5	Normal LD_1	Main	5.4 a	5.2	5.0-5.9	4.50-4.58
	Calcutta LD_1	Cathodal	5.4 a			
			5.3 a			
			5.2 a			
			5.1 a			
		Anodal	5.0 a			
	Beef LD_1	Main	5.8 a			
4-6.5	Normal LD_1	Main	5.20b			
	Calcutta LD_1	Cathodal	5.20a			
			5.08a			
			5.00a			
		Main	4.92c			

a. mean of 3 measurements
b. mean of 5 measurements
c. mean of 7 measurements

3.2.5 Electrophoretic demonstration of enzyme preparation purity

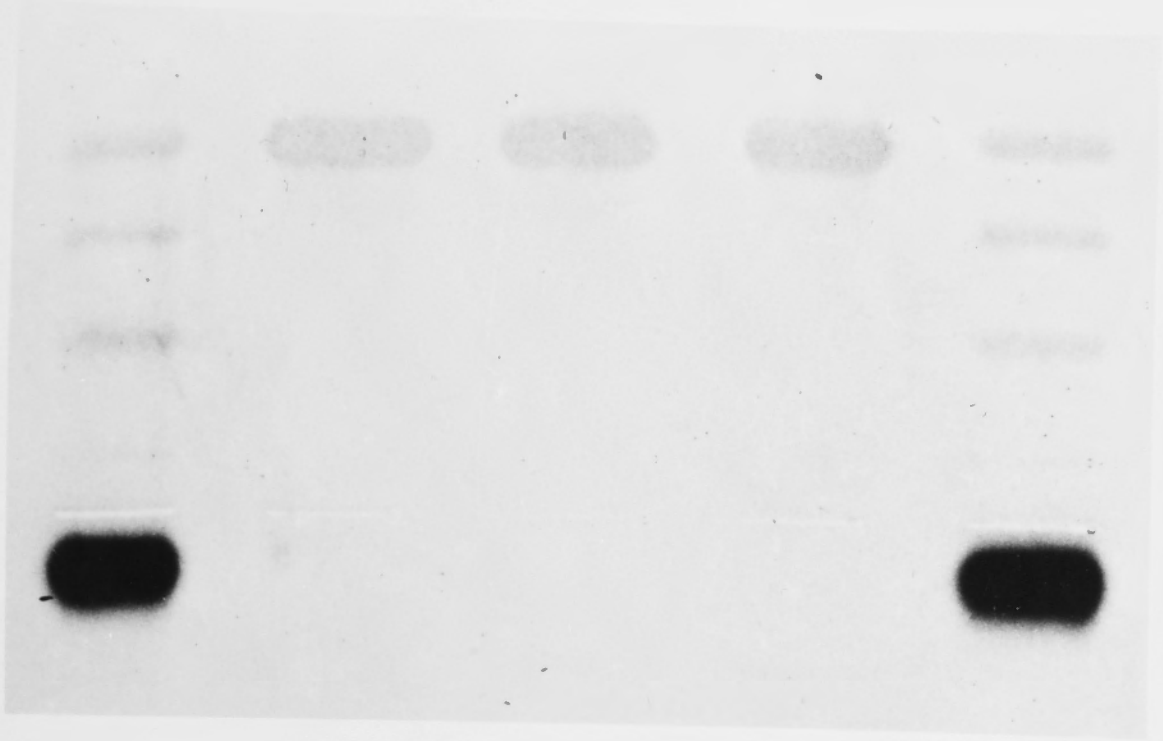
Purified enzyme preparations were subjected to the routine starch gel method at pH 7.0 and stained for enzyme to demonstrate that the preparation was free from the other LDH isoenzymes. Examples of purified LD_1 and LD_5 enzymes on such gels are shown in Figure 3.8.a,b respectively. Polyacrylamide gels were used for determining the protein purity of enzyme preparations at the later stages of purification. Both slab and disc techniques were utilized with acrylamide (AM-9) concentrations between 4% and 18%. Denaturing gels containing SDS or urea for the comparison of the polypeptide chains were also used. Gel compositions and buffer systems are listed in Appendix-2.

Electrophoresis was carried out in acrylamide slab gels, 0.2 cm × 14 cm × 17 cm, formed between glass plates, in a vertical electrophoresis apparatus manufactured by Raven Scientific Ltd., Haverhill, United Kingdom. The buffer system used was tris-glycine pH 8.9, see Appendix-2. Protein samples of 50-100 µg were applied in sucrose to the gel slots. A mixture of proteins as standards including bovine serum albumin, ovalbumin, ovomucoid and lysozyme in a solution containing urea and bromophenol blue tracking dye was included as a control. After 4 hours running at 250V, 20-45 mA, the gel was removed, stained in 0.2% Coomassie Blue in methanol/acetic acid/water for 30 min. and destained overnight in the same wash solution. Examples of these gels are shown in Figure 3.9 b,c,d,e. Figure 3.10 depicts the

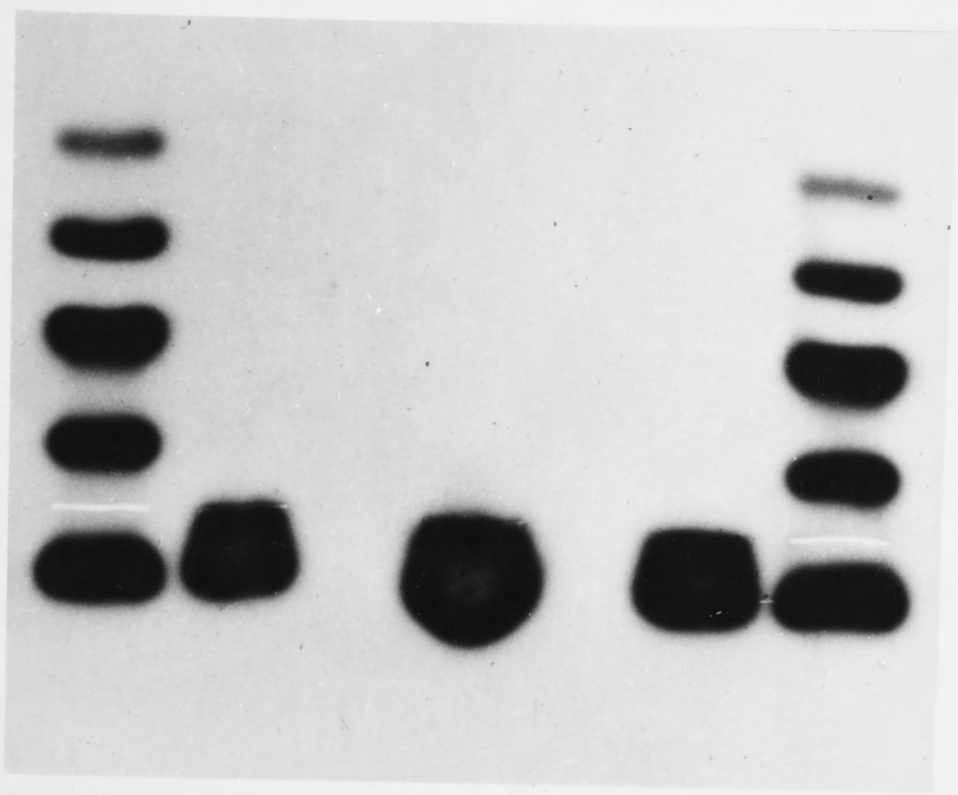
Figure 3.8 Purified isoenzymes, starch gel pH 7.0 stained for enzyme. Origin (O), anode at the top.

a. left to right, haemolysate control, 3 samples of purified LD_1 normal, control. (Hb cathodal to origin).

b. placental extract control, purified LD_5 normal, Calcutta LD_5, normal, control.



a



b

Figure 3.9 Purified isoenzymes and standards. Acrylamide slab SDS gels and disc gels (no SDS). Anode at the bottom, origin at the top. Stained for protein

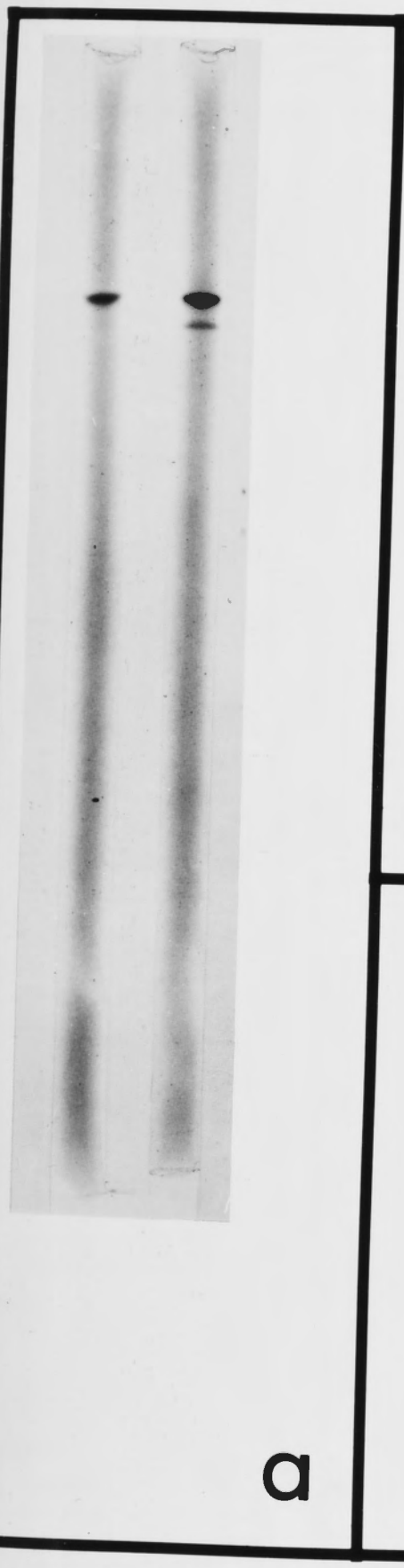
a. Disc gel, purified normal LD_1, earlier purification stage.

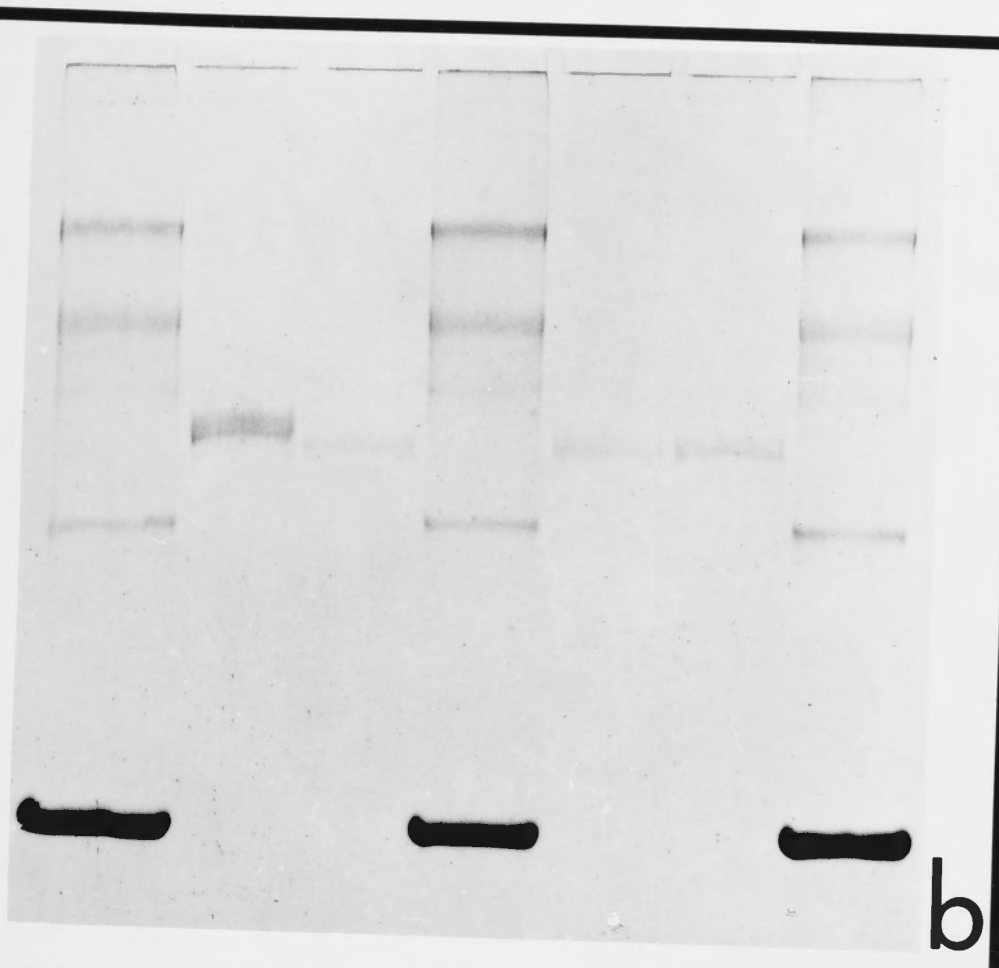
b. Slab gel, standards, purified Calcutta heterozygote LD_1 at various loadings.

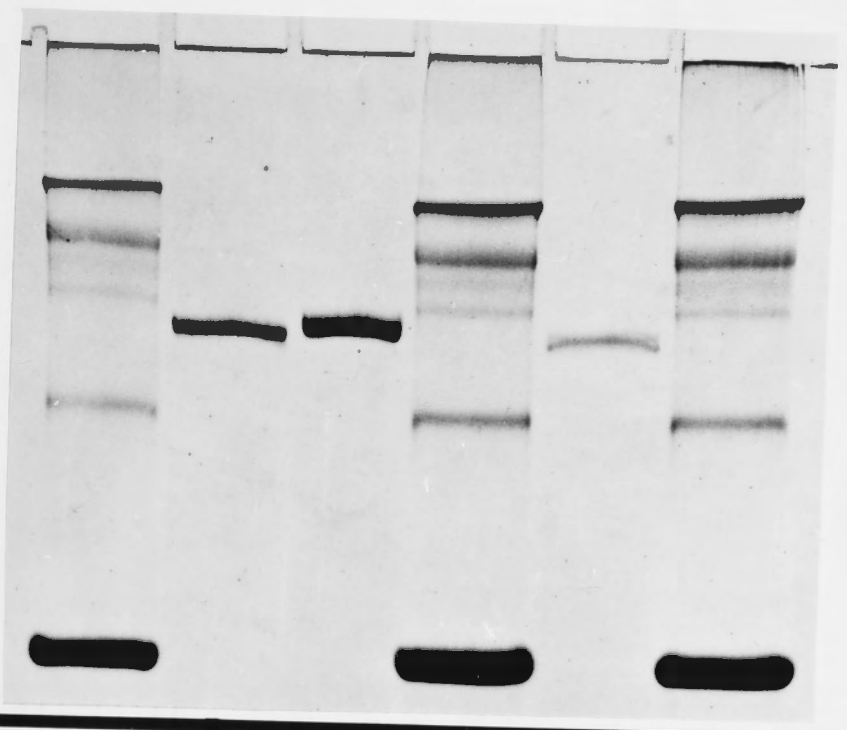
c. Slab gel, standards, purified normal LD_1 at various loadings.

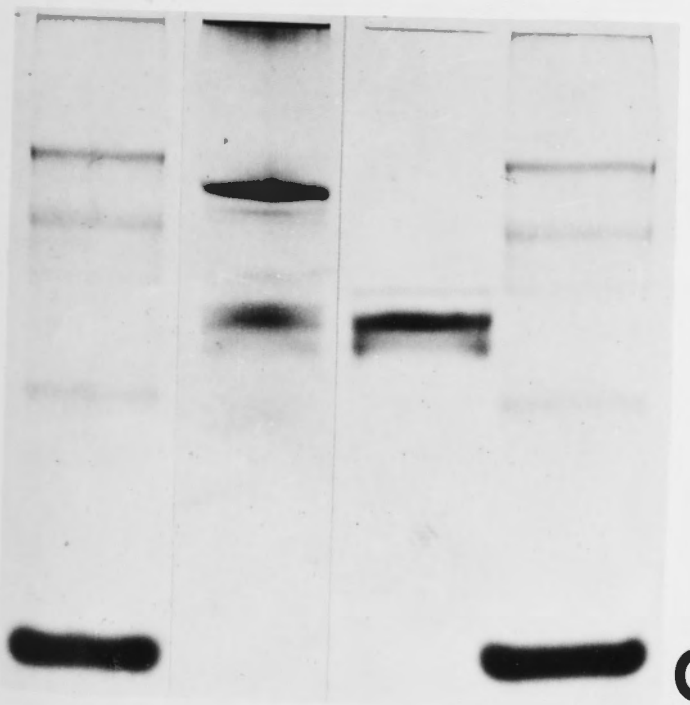
d. Slab gel, standards, purification stages of normal LD5.

e. Slab gel, normal LD_5 purified, normal LD_5, standards, normal LD_5, Calcutta LD_5, Calcutta LD_5.


a


b


c


d

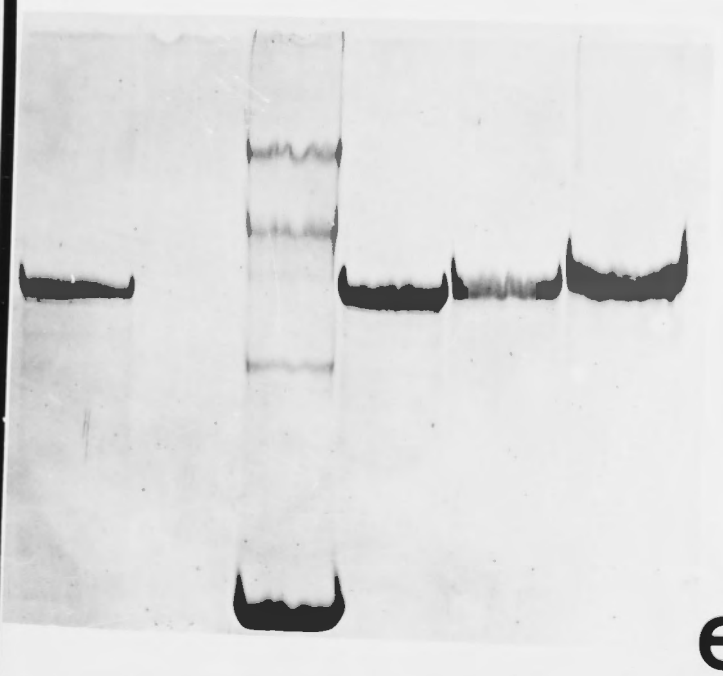
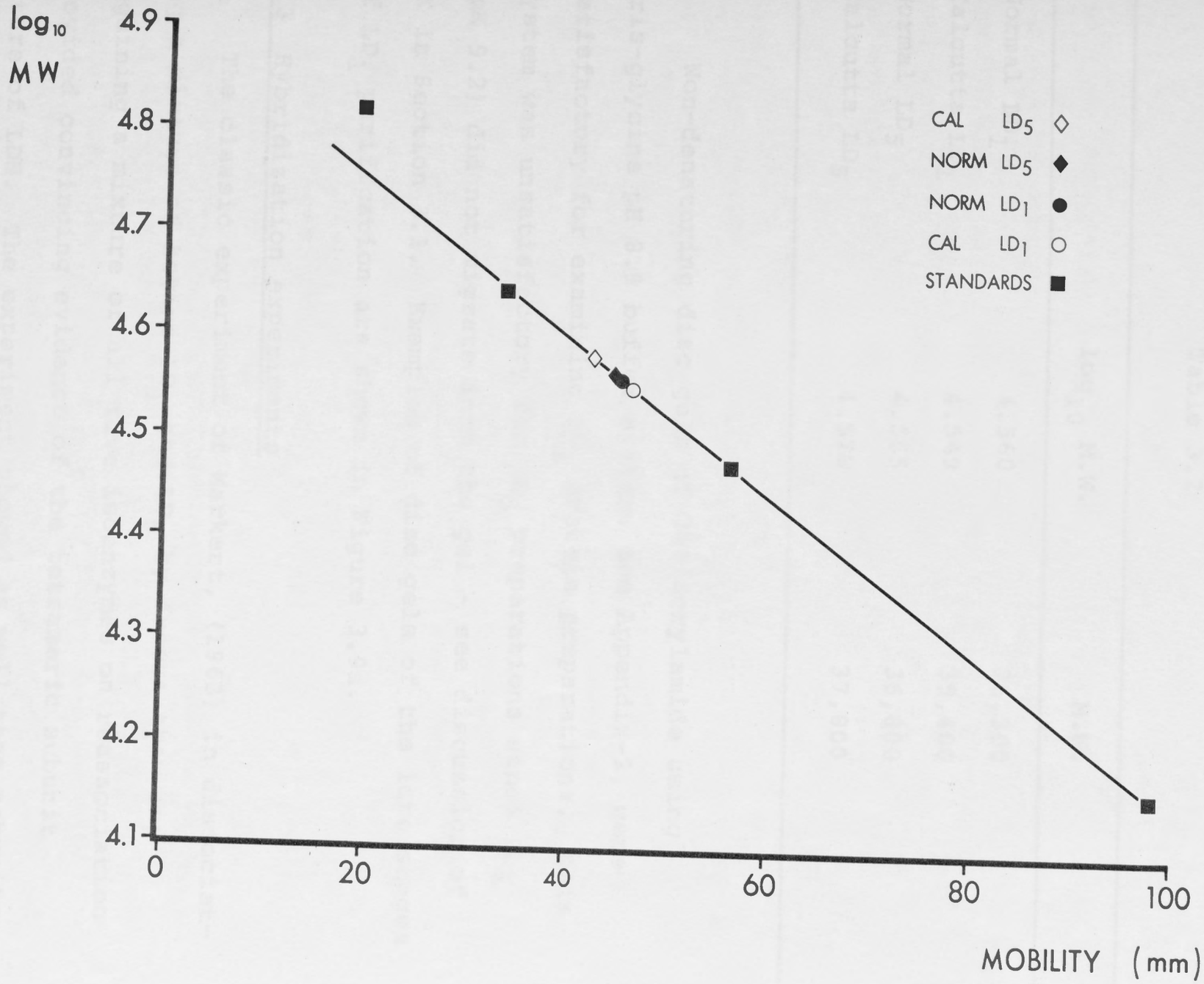

e

Figure 3.10 $\underline{\text{Log}_{10}\ \text{Molecular weight}}$ of standard proteins versus mobility on SDS-acrylamide slab gel. Purified LD_1 and LD_5 from Calcutta and normal.


log10 MW
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
0
20
40
60
80
100
MOBILITY (mm)
CAL LD5
NORM LD5
NORM LD1
CAL LD1
STANDARDS

regression line drawn through a plot of $\log_{10}$ M.W. of the protein standards versus their mobility from the origin. Entering the mobility values of the Calcutta and normal LDH's on this line yielded the M.W. values tabled in Table 3.2.

Table 3.2

	$\log_{10}$ M.W.	M.W.
Normal LD_1	4.560	36,300
Calcutta LD_1	4.549	35,400
Normal LD_5	4.565	36,800
Calcutta LD_5	4.578	37,800

Non-denaturing disc gels of 7½% acrylamide using tris-glycine pH 8.9 buffer system, see Appendix-2, were satisfactory for examining LD_1 protein preparations. This system was unsatisfactory for LD_5 preparations since LD_5 (pK 9.2) did not migrate into the gel - see discussion of pK in Section 3.1. Examples of disc gels of the late stages of LD_1 purification are shown in Figure 3.9a.

3.3 Hybridization experiments

The classic experiment of Markert, (1963) in dissociating a mixture of bovine LD_1 and LD_5 by freeze-thaw and regaining a mixture of all five isoenzymes on reassociation provided convincing evidence of the tetrameric subunit nature of LDH. The experiment showed as well that subunits could detach from the parent tetramer, and form new hybrid

tetramers which were enzymically active. Several authors have been able to repeat the experiment using LDH from different animals and techniques varying in detail but similar in principle (Chilson et al., 1964; Markert & Massaro, 1968; Millar et al., 1971; Jaenicke et al., 1971).

Hybridization experiments between Calcutta LD_1 and normal LD_5 or between Calcutta LD_5 and normal LD_1 should yield an isoenzyme banding pattern similar to that of the Calcutta heterozygote i.e. variant fast anodal isoenzyme bands at the intermediate isoenzymes. The appropriate hybridization of Calcutta with normal LDH should indicate whether the source of mutated subunits was A or B.

3.3.1 Experimental

A large number of hybridization trials with both purified and partly purified human LDH, with purified rabbit muscle LD_5 and with beef heart LD_1 were made using a range of conditions detailed in Table 3.3. Several of these experiments were successful in producing hybrid LD_3 and LD_4 between human LDH and rabbit LD_5, Figure 3.11.a,b,c.

Freeze/thaw methods generally resulted in low recovery of enzyme activity as can be seen in Figure 3.11,c, the left hand three tracks (freeze/thaw) compared with the three right hand tracks (Jaenicke method).

Early attempts at hybridization by the methods of Millar et al. (1971) and of Jaenicke et al. (1971) with partly purified human LD_1 and human LD_5 see Figure 3.11.d,e, did not yield the intermediate isoenzymes by hybridization. The method of Markert & Massaro (1968) using partly purified

Table 3.3 HYBRIDIZATION METHODS

METHOD	[PROTEIN] mg/ml	TEMP. °C	TIME HR	[NaCl] M	PHOSPHATE BUFFER M	pH	DTT mM
1. CHILSON et al. (1964)	2 -6.7	4	24	Saturated ∿6	0.1	7.0	-
2. MARKERT & MASSARO freeze/thaw (1968)	0.5	-20	66½	-	0.1	7.0	-
3. MILLAR et al. (1971)	5-50 0.005-.05	37	1	0.1	0.001-0.3	7.0 -9.0	5
4. JAENICKE et al. (1971)	0.03-3	4	24	4.6	0.067	5.6	0-5
5. This work.	0.5-5	-196	1	0 to Saturated	0.1	7.0	1-5

Figure 3.11 Hybridization experiments. Starch gels, pH 7.0 (4 hr), stained for enzyme, anode at the top of each zymogram.

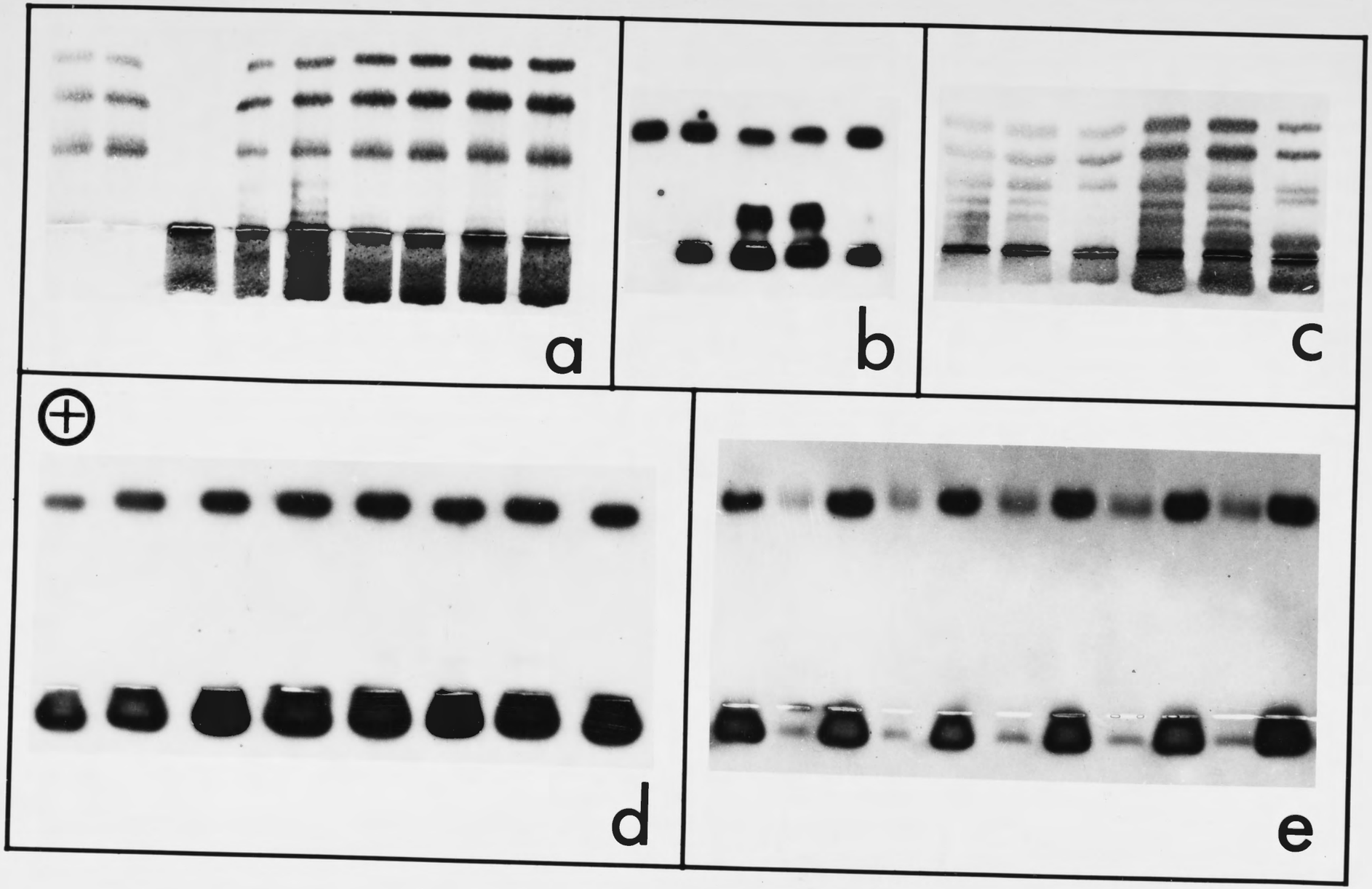
a. From left, human partly purified LDH from haemolysate (control), rabbit muscle LD_5 (control), Chilson et al. (1964) method.

b. Human LD_1, control mixture of human LD_1 rabbit LD_5, hybridized mixture, replicate, control mixture. Jaenicke et al. (1971) method.

c. Partly purified human haemolysate with rabbit LD_5, first 3 samples freeze/thaw method of Markert & Massaro (1968) dilutions of protein 2.7, 0.54, 0.27 mg/ml Second 3 samples same sample (2.7 mg/ml) 4°, 26°, 37°. Note relative reduction in overall activity of freeze/thaw method cf. Jaenicke method.

d. Partly purified human LD_1 and human LD_5 hybridization attempt by Millar et al. (1971) method.

e. Partly purified human LD_1 and human LD_5 hybridization attempt by Jaenicke et al. (1971) method, alternately 3.0 and 0.3 mg/ml mixtures.


a
b
c
d
e

human haemolysate and purified rabbit LD_5 produced some hybrid bands, Figure 3.11.c, but with substantially lower activity in all isoenzyme bands. This method involves slow freezing of the starting material to -20°C in 0.1 M phosphate buffer at pH 7.0 with no NaCl added. When the hybridization mixture was thawed, the hybrid isoenzymes were demonstrated by gel electrophoresis.

The Markert & Massaro (1968) method was followed exactly using unpurified human haemolysate and human liver homogenate mixtures and the result is summarized in Table 3.4.A. To overcome the resulting loss of activity the method was further developed along the following lines. Protein concentration was increased from 0.5 mg/ml to 5 mg/ml, and dithiothreitol (DTT) 1-5 mM was added. The results of this experiment appear as B in Table 3.4. To increase the dissociation and reassociation process, 1-4 cycles of rapid freeze/thaw to liquid nitrogen temperature (-196°C) followed by thawing to room temperature (20°C) experiment C, or to melting ice temperature (0°C) experiment D. Activity was better preserved in these experiments especially in D, but no hybridization as judged by increased LD_3 activity relative to the other bands was observed.

In experiment E, NaCl was added to give 1 M, 3 M, 5 M and saturated (approximately 7 M), final concentration and the hybridization mixtures were subjected to 3 cycles of liquid nitrogen to 0°C freeze/thaw. Each cycle took about 20 minutes to complete. The controls and the control mixtures were held at 4°C (all experiments) until required for electrophoresis. The hybridization mixtures were dialysed overnight

Table 3.4 HYBRIDIZATION RESULTS

Experiment Normal human tissues	Iso-zyme	LD_1 rich contr.	LD_5 rich contr.	Control mix	Isoenzyme content after treatment.	Results
A						
F/T -20°	LD_1	+++	tr	+++	++	Activity lost overall, mostly in A-rich isoenzymes.
0.5mg/ml	LD_2	+++	tr	+++	++	
	LD_3	++	+	+++	+	
	LD_4	tr	+++	+++	tr	
	LD_5	-	+++	+++	-	
B						
F/T -20°	LD_1	+++	tr	+++	+++	Less activity loss than A No hybridizat-ion.
5mg/ml	LD_2	+++	tr	+++	++	
1-5mM DTT	LD_3	++	+	+++	++	
	LD_4	tr	+++	+++	+	
	LD_5	-	+++	+++	+	
C						
F/T liq.N_2 to 20°	LD_1	+++	tr	+++	++	Activity loss at LD_3-LD_5 No hybridization
one cycle	LD_2	+++	tr	+++	++	
5mg/ml	LD_3	++	+	+++	+	
5mM DTT	LD_4	tr	+++	+++	+	
	LD_5	-	+++	+++	tr	
D						
F/T liq.N_2 to 0°	LD_1	+++	tr	+++	+++	Less activity loss than in C, possibly hybridization.
3 cycles	LD_2	+++	tr	+++	+++	
5mg/ml	LD_3	++	+	+++	+++	
5mM DTT	LD_4	tr	+++	+++	++	
	LD_5	-	+++	+++	+	

Experiment	Iso-zyme	LD_1 rich contr.	LD_5 rich contr.	Control mix	1M	3M	5M	7M	Results
E					NaCl conc.				
F/T liq.N_2 to 0°	LD_1	+++	tr	+++	tr	tr	tr	tr	LD_1, LD_2 loss, probable hybridization at LD_3.
3 cycles	LD_2	+++	tr	+++	tr	tr	tr	-	
5mg/ml	LD_3	++	+	+++	++	+++	+++	+++	
5mM DTT	LD_4	tr	+++	+++	+	+	++	++	
	LD_5	-	+++	+++	++	++	tr	tr	

Footnote: Experiments A-E carried out with crude haemolysate and crude liver homogenate, both from normal human tissues. F/T = freeze/thaw 5mg/ml = protein conc. DTT = dithiothreitol.

Table 3.4 (contd.) HYBRIDIZATION RESULTS

Experiment	Iso-zyme	LD_1 rich contr.	LD_5 rich contr.	Control mix	Isoenzyme content after treatment		Results
					5M	7M	
Heat treated normal haemolysate and DEAE treated liver homogenate.							
F as for E.	LD_1	+++	-	+++	tr	-	NaCl conc. Overall activity loss, hybridization at LD_3.
	LD_2	+	tr	+	-	-	
	LD_3	tr	tr	+	++	++	
	LD_4	-	++	++	+	+	
	LD_5	-	+++	+++	tr	+	
Crude Calcutta-het. haemolysate and DEAE treated liver homogenate.							
G as for E.	LD_1	+++	-	+++	+	tr	Activity loss, ? hybridization at LD_3. No variant bands.
	LD_2	+++*	tr	+++*	+	-	
	LD_3	++*	tr	++*	+	+	
	LD_4	tr	++	++	+	+	
	LD_5	-	+++	+++	+	tr	
Partly purified Calcutta-het. LD_1 and DEAE treated liver homogenate.							
H as for E.	LD_1	+++	-	+++	+	tr	Activity loss, hybridization at LD_3, no variant bands at LD_3 or LD_4.
	LD_2	+*	tr	+*	-	-	
	LD_3	-	tr	tr	+	+	
	LD_4	-	++	++	+	+	
	LD_5	-	+++	+++	+	+	
1-5mM NAD, NADH, 1-10% glycerol.							
I as for E.	LD_1	+++	-	+++	+++	+++	Activity preserved especially LD_1 and LD_2, no variant hybrid bands.
	LD_2	+*	tr	+*	+	+	
	LD_3	-	tr	tr	+	+	
	LD_4	-	++	++	+	+	
	LD_5	-	+++	+++	++	+	

Footnote: Experiment G-I carried out with Calcutta-het. material from blood. Starting material in I same as for H. * = variant band(s) present.

at 4°C against 5 litres of 0.1 M phosphate pH 7.0, 1 mM DTT to reduce the salt concentration. The samples and controls were then examined by starch gel electrophoresis. Desalting was essential, since unsatisfactory electrophoretic separation of the isoenzyme bands resulted if the samples were not desalted. The results of E suggested that hybridization might be occurring under these fairly harsh conditions. The maintenance of the relative amount of LD_3 suggested that this band was being produced by hybridization at the expense of the other bands, especially at higher salt concentrations. The use of mixtures of crude haemolysate and liver homogenate, while satisfactory in terms of amount of total activity and higher protein concentration, held the distinct disadvantage of presenting an extremely generalised isoenzyme banding pattern in the control mixture. With this material, increases in LD_3 and LD_4 by hybridization were difficult to discern. Superimposed on this was the lower stability to extreme conditions of the A-rich isoenzymes LD_4 and LD_5, resulting in the probable reduction of these isoenzymes by inactivation rather than by hybridizational loss of subunits to other isoenzyme forms.

The method was therefore extended to using partly purified LDH. LD_1 material was conveniently produced by heating a 10 ml aliquot of normal blood to 68°C for 10 minutes followed by centrifugation at 10,000 g. This treatment significantly increased the LD_1 proportion of the total LDH activity, Figure 4.8. LD_5-rich material was produced by passage of the centrifuged liver homogenate through a DEAE-Sephadex column, Figure 2.4, followed by concentration

of the eluted enzyme. The altered isoenzyme content of these starting materials is indicated in Table 3.4 experiment F. The same 3 cycle freeze/thaw regime was followed using this new starting material. The results of this hybridization are shown in Table 3.4.F. The activity of LD_3 increased in relation to the other isoenzymes and to the control, showing that hybridization had occurred.

Hybridization of Calcutta-1 heterozygote LD_1

Heterozygote Calcutta haemolysate was added to unpurified normal liver homogenate and subjected to the hybridization method of experiment E. This experiment is G in Table 3.4. Variant bands in LD_2 and LD_3 were not observed after hybridization. Although the LD_1 band was decreased after treatment it could not be concluded that LD_3 bands, hybrid or normal, were formed at the expense of LD_1 in the starting material.

Heat purification of Calcutta haemolysate was not used to produce an LD_1-rich starting material for hybridization experiments in case thermally induced impairment of hybridizability might result. Instead variant haemolysate was partly purified by Blue Dextran chromatography. This enzyme material was concentrated and made up to 5 mg/ml with bovine serum albumin. The presence of other proteins was not considered to affect LDH hybridization (Jaenicke et al. 1971). Experiment H utilized this partly purified variant LD_1 and was mixed with partly purified LD_5 from normal human liver. The variant bands present in the control mixture in LD_2 were not observed in bands LD_3 or LD_4 on the

proving gel after hybridization treatment. In no experiment was it found possible to produce hybrid variant bands in any of the isoenzymes. A number of combinations of NAD, NADH and glycerol with the experimental E conditions were tried, to preserve activity in the Calcutta hybridizations. These are grouped in summary in experiment I, Table 3.4. In all cases hybridization appeared to be inhibited although the protection of activity was promoted.

The hybridization experiments performed indicate that a measure of hybridization can be achieved with human LDH using freeze/thaw conditions seemingly harsher than other authors have found necessary with other animal LDH's. The method has not led to a useful result when applied to variant material. Some bands of Calcutta LDH, at least, are heat labile (Chapter 4) and are labile to storage. While hybridization of B and β subunits from LD_1 tetramers to LD_2, LD_3 amd LD_4 might well have resulted from the hybridization conditions used, they could not be demonstrated by activity staining on starch gels. It might be reasonable to expect mutant subunits to hybridize under the same conditions that normal subunits do. However this could not be demonstrated. It is possible the harsh conditions of high salt, freeze/thaw reduces the mutant LDH activity below the demonstrable level by gel staining, even though hybridized protein might be present.

3.4 Discussion

All of the B-containing variant isoenzymes including LD_1 possess faster anodal migrating bands than the

equivalent normal isoenzymes and this effect is independent of the buffer composition, concentration or pH. Calcutta LD_5 always migrated as a single band with variable mobility. In some buffer systems the Calcutta-1 was faster anodally, in some slower and in one system possessed identical migration with normal. This variability in migration is not consistent with an anodally fast charge mutation in the A subunit. The absence of multiple bands for variant heterozygote LD_5 in these zymograms as well as the observation of only a single band with the same migration as normal on the protein-stained, urea denaturing gel is inconsistent with Calcutta-1 being an A variant.

On the other hand, the comparison of highly purified normal LD_1 with Calcutta heterozygote LD_1 by the method of isoelectric focusing and protein staining clearly demonstrates multiple banding of the variant protein. At least five protein bands differing from each other by 0.1 pI units were found by this technique. The variant LD_1 possess a band with the same migration as the principal normal LD_1 band, pI = 5.4. That the Calcutta LD_1 could be separated into two bands on dissociating urea/acrylamide gels, whereas normal LD_1 yielded only a single band under the same conditions, is further evidence of a B-subunit mutation. It is suggested that the two bands found for Calcutta heterozygote LD_1 are the normal B polypeptide subunit and an altered mutant β polypeptide of faster anodal migration.

The multiple banded pattern observed on electrofocusing of pure Calcutta LD_1 is consistent with the five possible tetrameric associations of B and β subunits introduced in Chapter 1, Figure 1.3.c. The overall conclusion drawn from the various electrophoretic approaches is that Calcutta-1 LD_1 is a fast B mutation.

The method employed in Figure 3.10 to estimate the molecular weights of the LDH subunits is certainly insufficiently precise to obtain information at the individual amino-acid residue level (assuming a mean residue weight of ∿ 105). Within the inherent experimental errors of the method however, the data does suggest that subunits close to the accepted subunit size of 35,000 (Holbrook et al., 1975) are involved.

4. THERMAL EFFECTS ON THE ENZYME'S ACTIVITY AND STRUCTURE

4.1 Kinetic effects - Arrhenius plots

The effects of thermal energy on enzymes may be arbitrarily separated into two categories (Laidler and Bunting, 1973). The first is the effect of temperature on the kinetics of the enzyme catalyzed reaction. As the temperature is raised, an increased rate of enzyme catalyzed reaction occurs, brought about by a change in the magnitude of the equilibrium constant. This effect of temperature may be described by the Arrhenius equation ...

$$k = Ae^{-Ea/RT}$$

where k is the overall rate constant, Ea is the Arrhenius activation energy, T is the absolute temperature in ^{o}K, A is an integration constant and R is the gas constant (Wong, 1975). The relationship may be rearranged to the differential form ...

$$Ea = - 2.303\ R \frac{d \log k}{d\ T^{-1}}$$

Since Vmax, the limiting or maximum velocity of the reaction is dependent on the overall rate constant, a plot of log Vmax against $\frac{1}{T}$ yields a straight line with slope of $\frac{Ea}{2.303\ R}$ (Dixon and Webb, 1959). The substitution of Vmax for k in

the equation changes the position but not the slope of the plot.

Arrhenius plots of vertebrate LDH's have been made using this method by Baldwin and Aleksiuk (1973) and by Somero and Low (1977). In the current work LDH reaction velocities were measured spectrophotometrically by the methods described in Chapter 5.

4.1.1 Experimental

Vmax and Km (pyruvate) values were determined from Lineweaver-Burk plots at the appropriate temperatures. At the start of each experiment, purified stock enzyme solutions were diluted into buffer containing 1 mg/ml BSA and the activities of the normal and variant enzymes adjusted to match by small additions of stock enzyme. This enzyme dilution contained 3.00 units/ml LDH. The assays were made at each temperature and pyruvate concentration, in quadruplicate using 0.015 units of enzyme per assay. The behaviour of reaction velocity as the assay temperature is increased at various concentrations of pyruvate is shown in Figure 4.1. An example of a Lineweaver-Burk plot used to determine Vmax and Km (pyruvate) data at temperatures from 20-50^{o}C is shown in Figure 4.2. The regression line was calculated using a minimum of four data points on the linear portion of the plot. The relationships between Km (pyruvate) and assay temperature for each pair of enzymes are plotted in Figures 4.3 and 4.4. The concentration of pyruvate required to attain half maximum reaction velocity rises rapidly with increased temperature.

Figure 4.1 $\frac{\text{\% Velocity versus temperature}}{\text{Velocity at 0.33 mM pyruvate, 30°C = 100\%.}}$ purified LD_5.

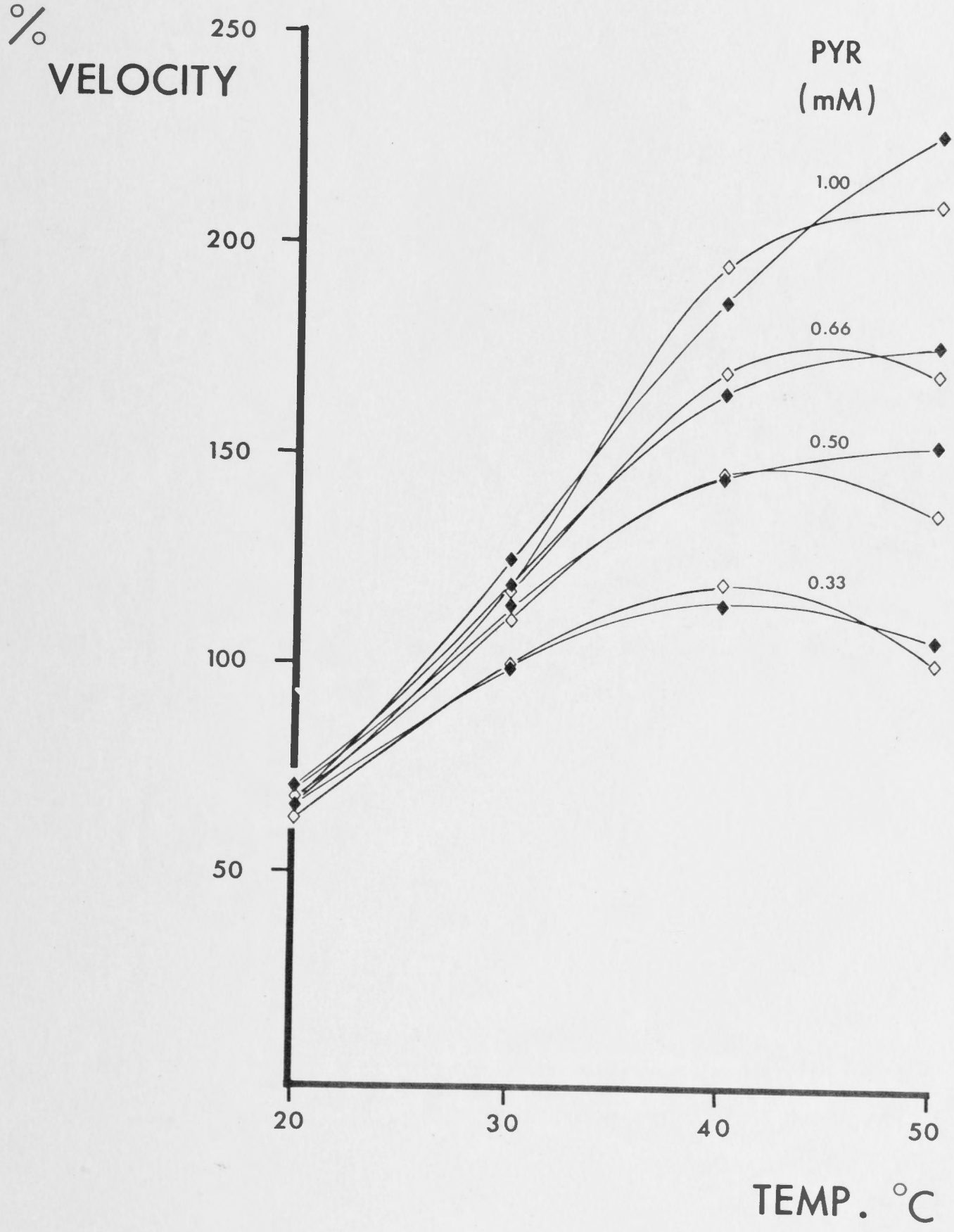

%
VELOCITY
250
200
150
100
50
PYR
(mM)
1.00
0.66
0.50
0.33
20
30
40
50
TEMP. °C
NORM LD5
CAL LD5

Figure 4.2 Lineweaver-Burk plots purified normal LD_5, $\frac{1}{v}$ units $(\Delta OD_{340})^{-1}$ min., $\frac{1}{pyr}$ units $(mM)^{-1}$.

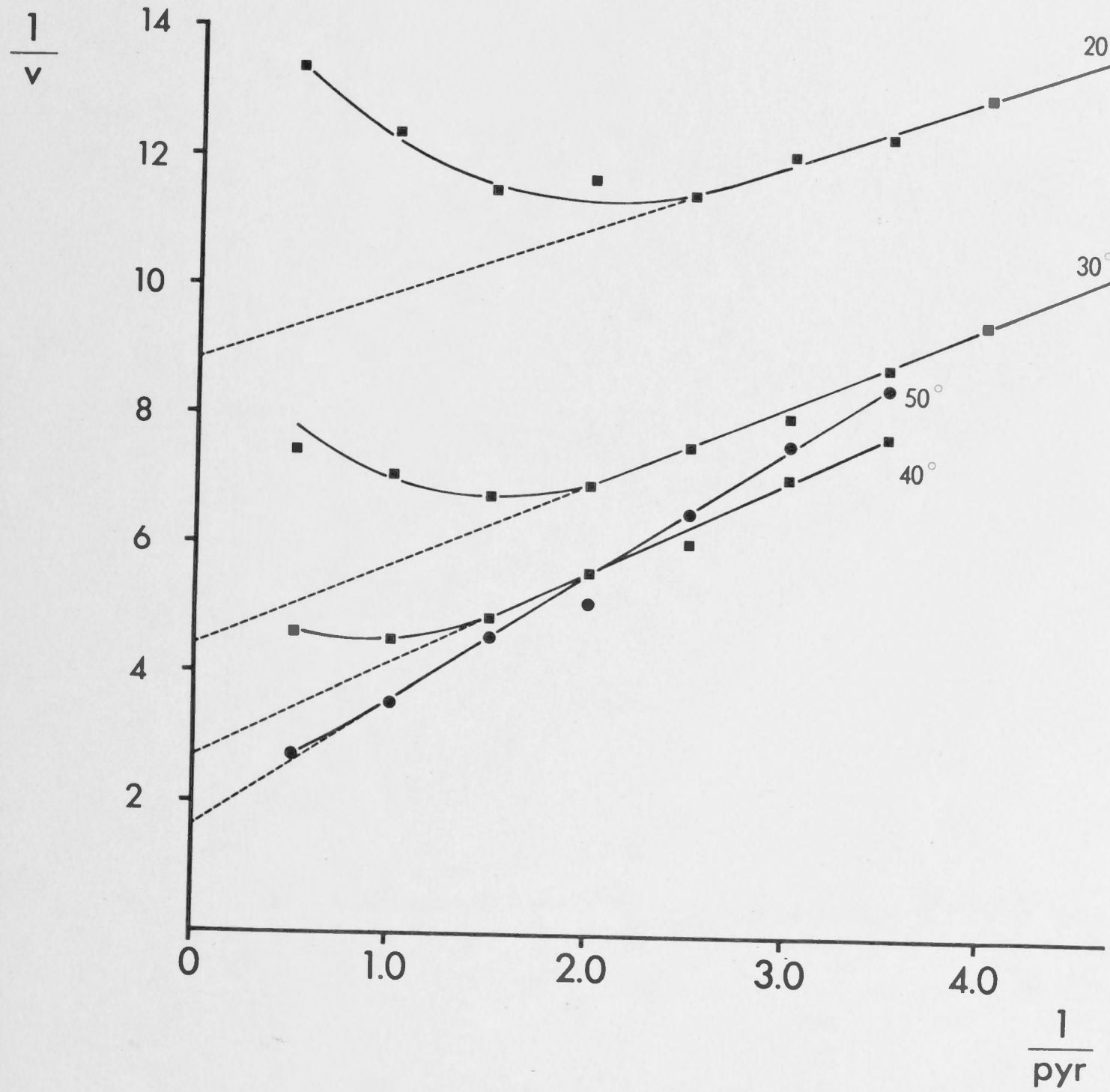

1/v
14
12
10
8
6
4
2
0
1.0
2.0
3.0
4.0
1/pyr
20°
30°
50°
40°

Figure 4.3 Km (pyruvate) versus temperature.
Haemolysates and purified LD_5.

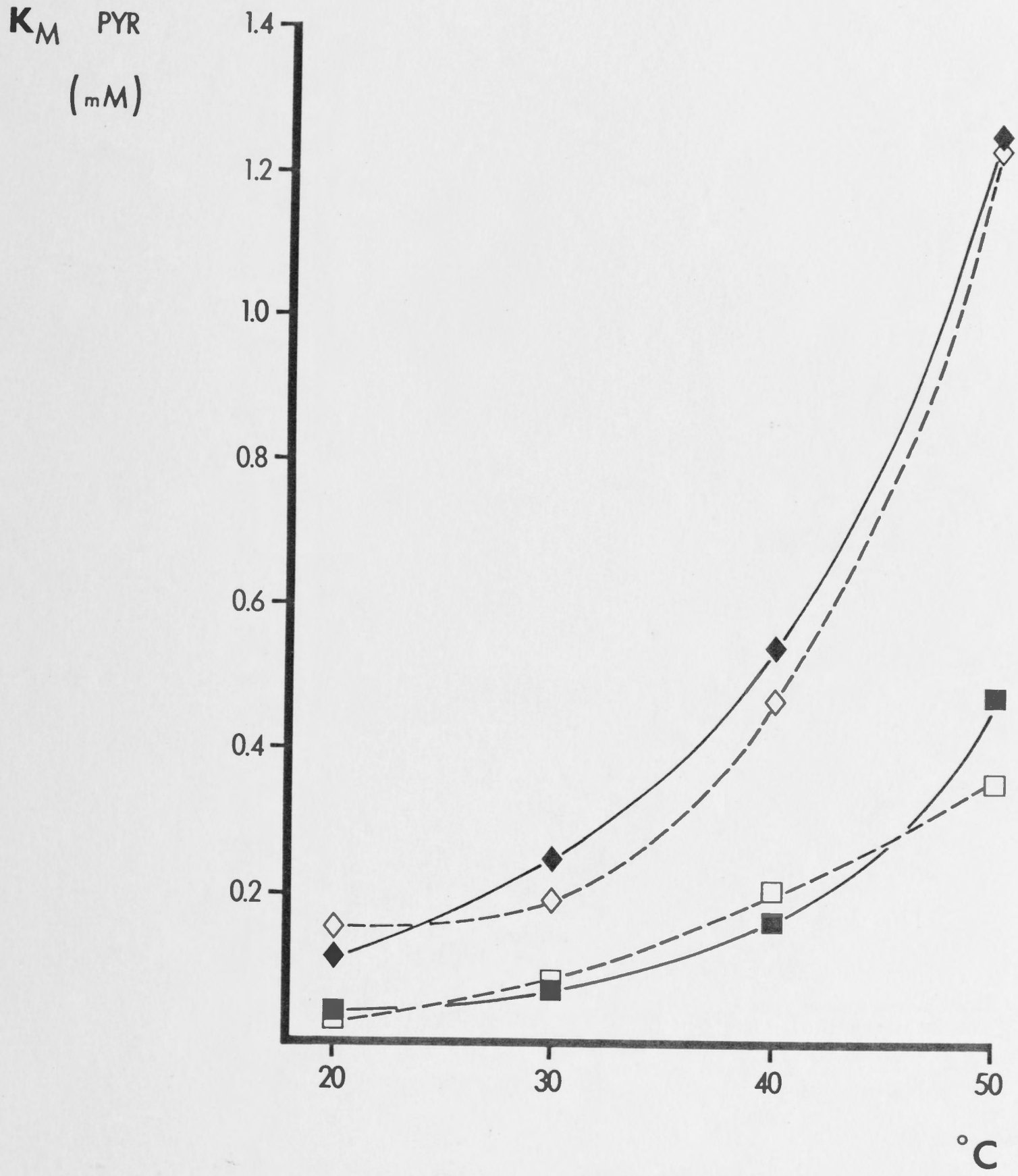
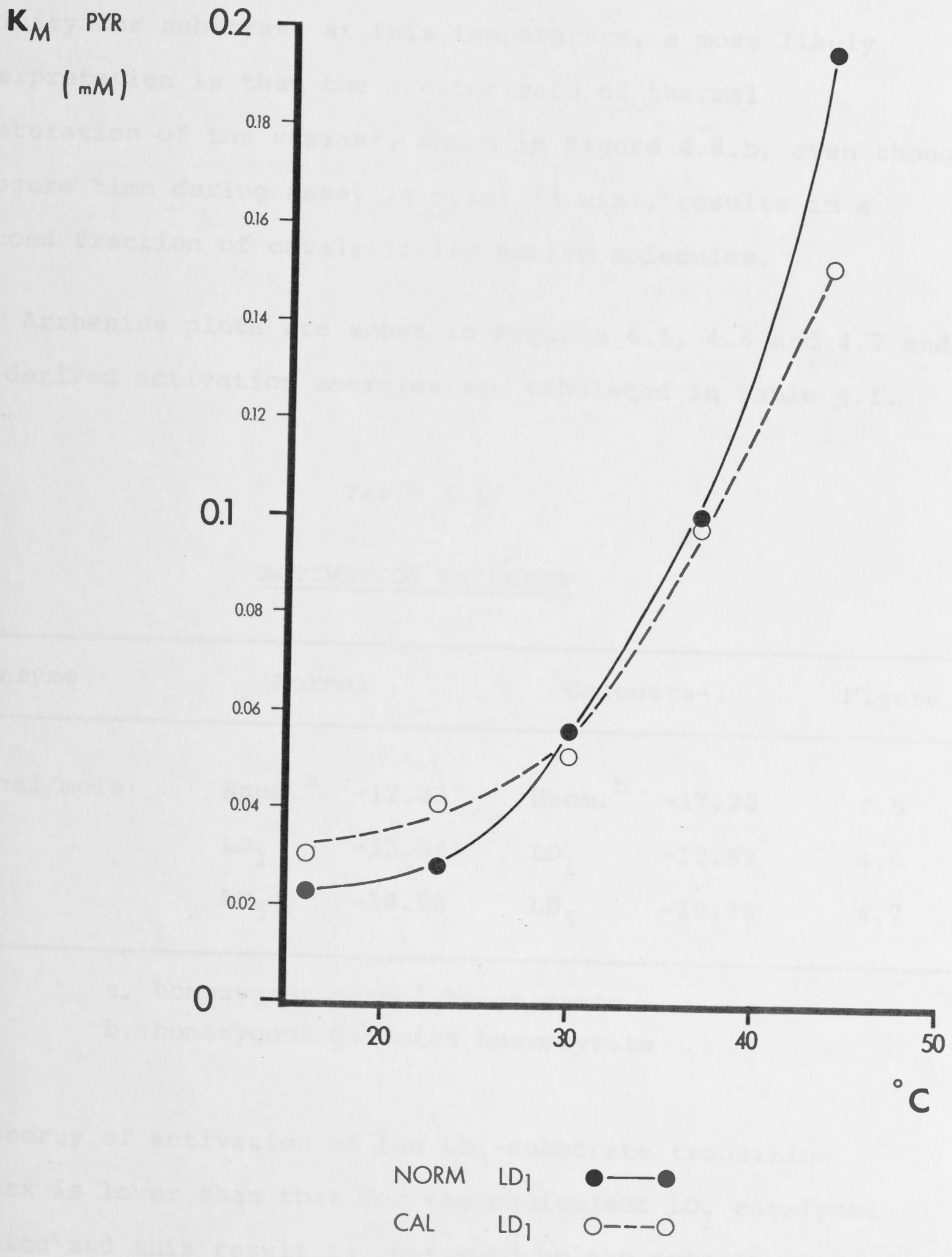

K_M PYR
(mM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
20
30
40
50
°C
NORM HAEMOLYSATE
CAL–HOM HAEMOLYSATE
CAL LD5
NORM LD5

Figure 4.4 Km (pyruvate) versus temperature purified LD_1. (N.B. expanded Km scale).


K_M PYR
(mM)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
20
30
40
50
°C
NORM LD1
CAL LD1

Calcutta and normal LD_5 behave similarly in this respect, as do Calcutta homozygote and normal haemolysate in the range 20^{o}-40^{o}C. While the apparent divergence in Km's at 50^{o}C (see Figure 4.3) might reflect a different thermally induced affinity for substrate at this temperature, a more likely interpretation is that the greater rate of thermal denaturation of the variant, shown in Figure 4.8.b, even though exposure time during assay is brief (3 min), results in a reduced fraction of catalytically active molecules.

Arrhenius plots are shown in Figures 4.5, 4.6 and 4.7 and the derived activation energies are tabulated in Table 4.1.

Table 4.1

ACTIVATION ENERGIES

Enzyme	Normal		Calcutta-1		Figure
Ea(Kcal/mole)	Haem.[a]	-12.23	Haem.[b]	-17.20	4.5
	LD_1	-13.04	LD_1	-12.69	4.6
	LD_5	-10.53	LD_5	-10.24	4.7

a. homozygous normal haemolysate
b. homozygous Calcutta haemolysate

The energy of activation of the LD_5-substrate transition complex is lower than that for the equivalent LD_1 catalysed reaction and this result is confirmed by the experimental figures obtained. The Arrhenius plots for purified LD_5's

Figure 4.5 Arrhenius plots, haemolysates. log V_{max} should be -ve units. Abscissa units $(°K)^{-1}$. Equivalent range 50-20°C. Possible denaturation at 40-50°C, plot is dotted.

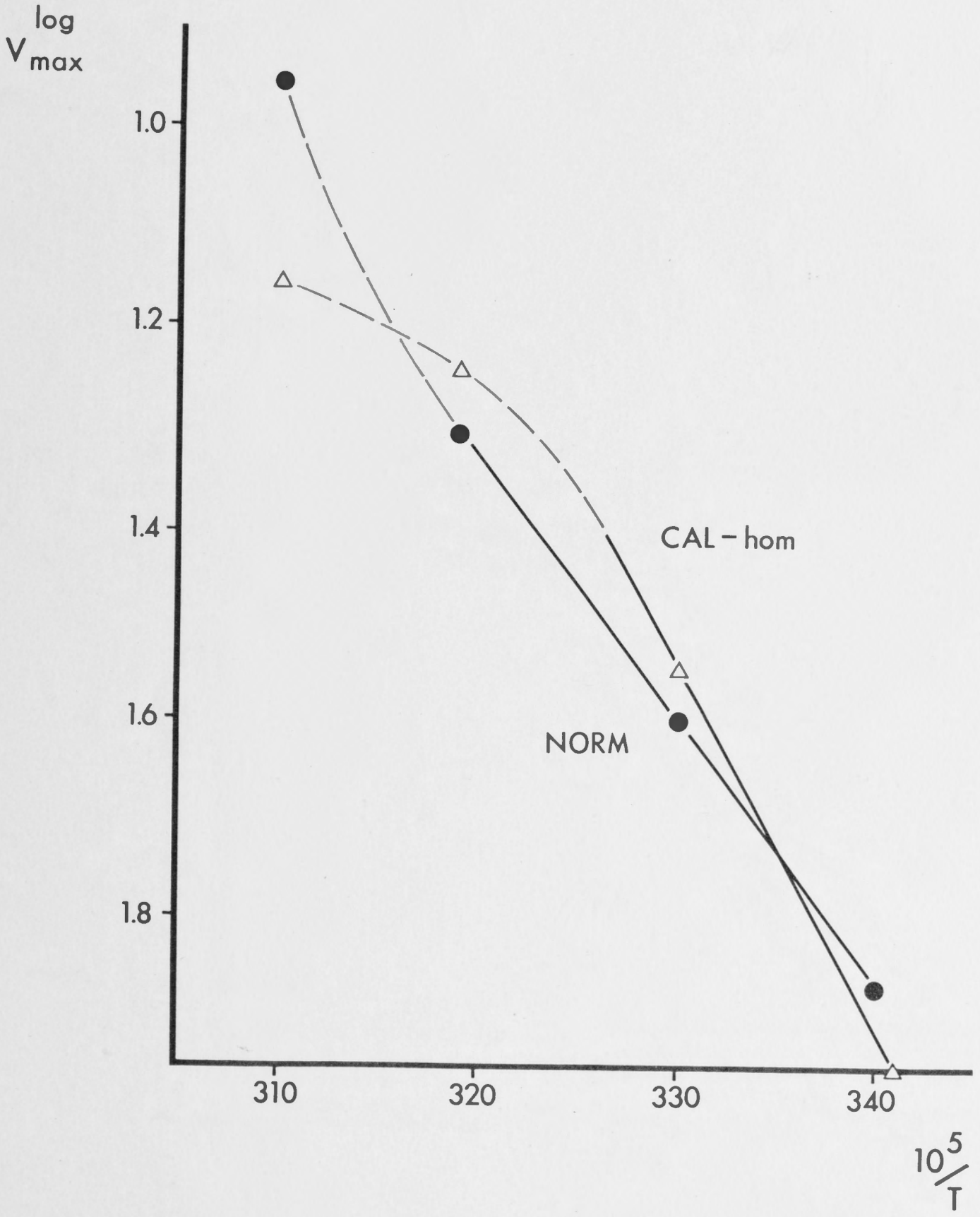

log
V_{max}
1.0
1.2
1.4
1.6
1.8
CAL – hom
NORM
310
320
330
340
$10^5/T$

Figure 4.6 Arrhenius plots, purified LD_1. Abscissa units $(°K)^{-1}$. Equivalent range 44-16°C. Determination at 16°C ∿ 346 $(°K)^{-1}$ less reliable, plot is therefore dotted.

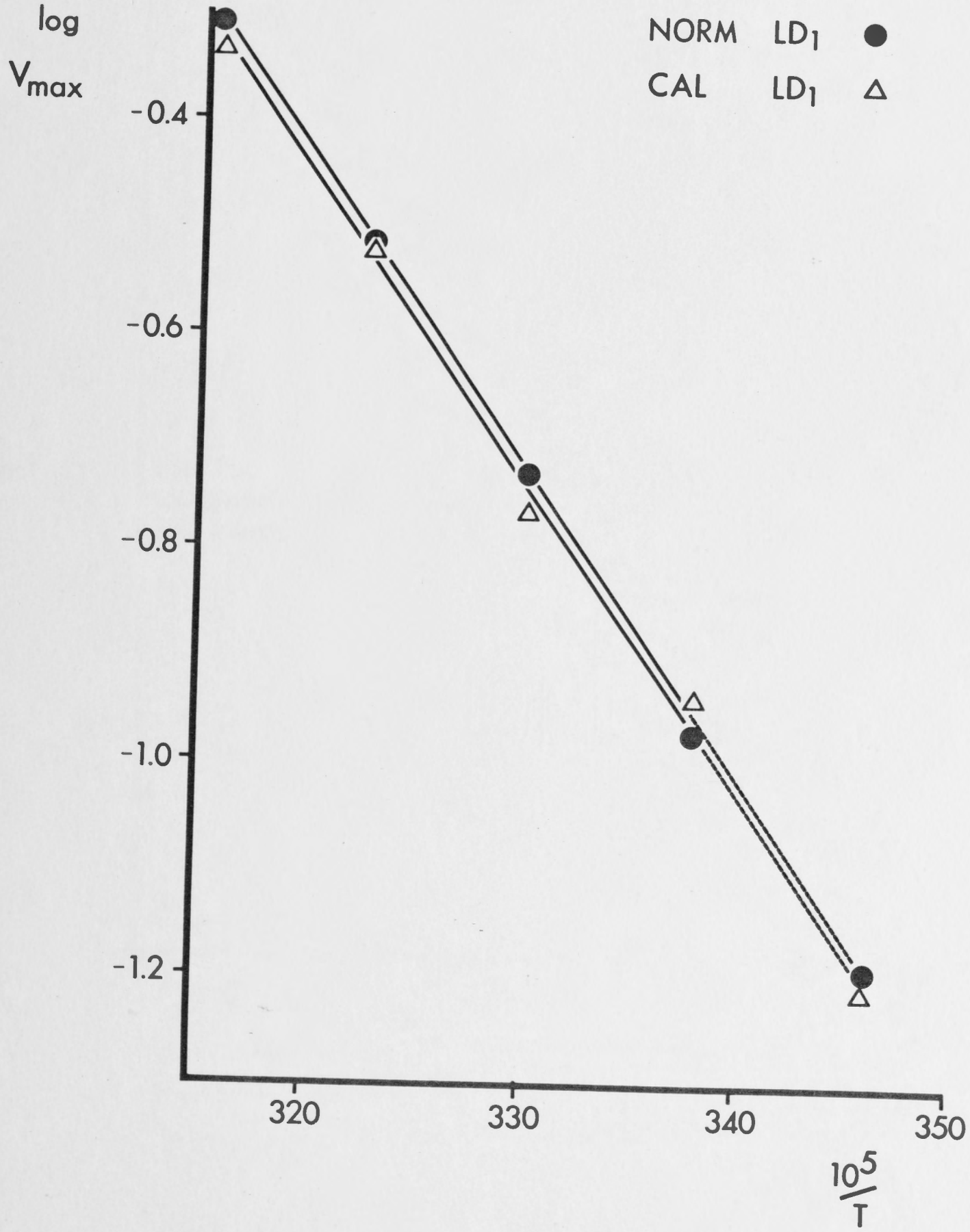
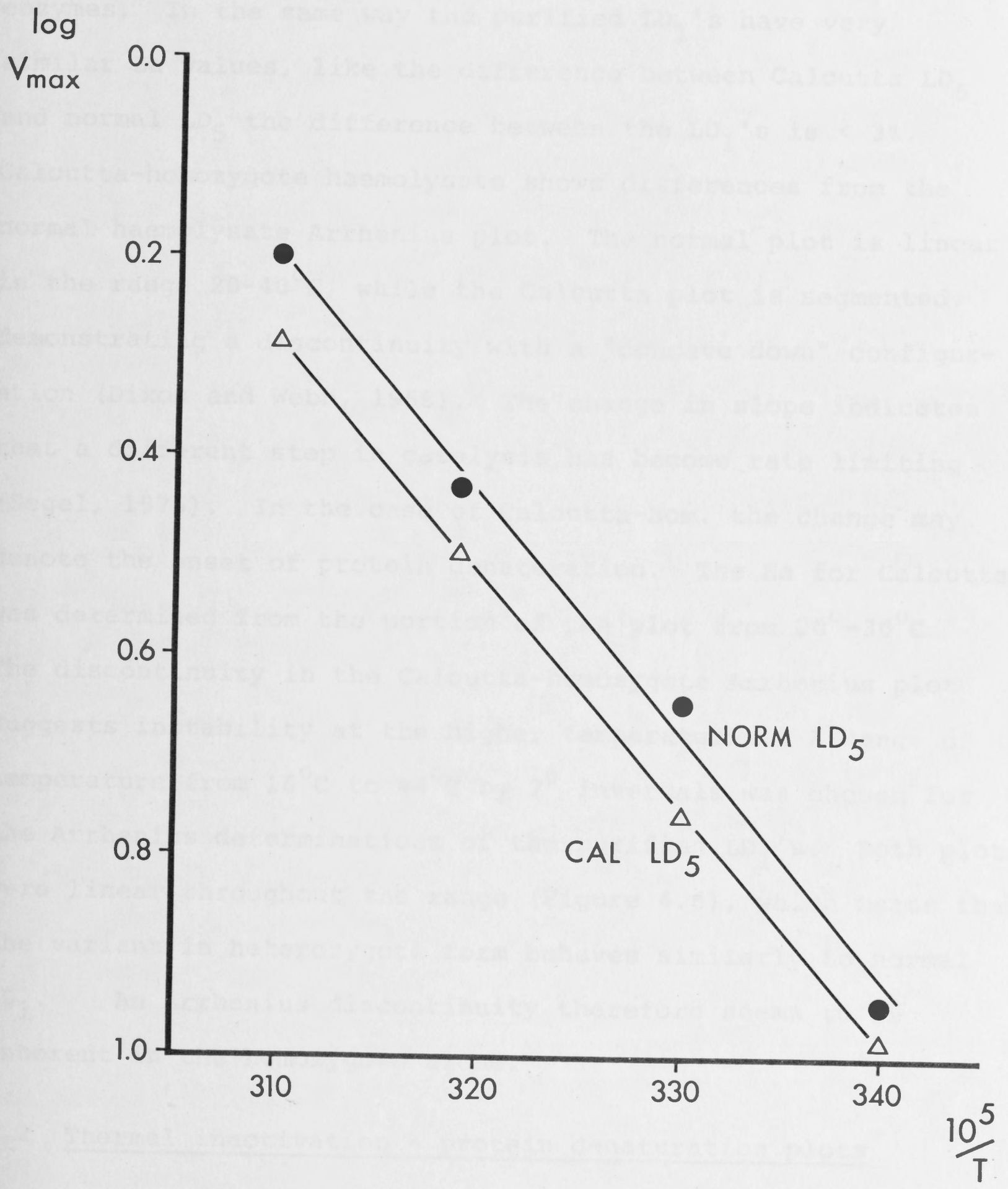

log
V_{max}
-0.4
-0.6
-0.8
-1.0
-1.2
320
330
340
350
$\frac{10^5}{T}$
NORM LD1
CAL LD1

Figure 4.7 Arrhenius plots, purified LD_5. log V_{max} should be -ve units. Abscissa units (°K)-1. Equivalent range 50-20°C.


log
V_{max}
0.0
0.2
0.4
0.6
0.8
1.0
310
320
330
340
$10^5/T$
NORM LD_5
CAL LD_5

both Calcutta and normal, together with the similar derived Ea values suggest little if any difference between these two enzymes. In the same way the purified LD_1's have very similar Ea values, like the difference between Calcutta LD_5 and normal LD_5 the difference between the LD_1's is < 3%. Calcutta-homozygote haemolysate shows differences from the normal haemolysate Arrhenius plot. The normal plot is linear in the range 20-40°C, while the Calcutta plot is segmented, demonstrating a discontinuity with a "concave down" configuration (Dixon and Webb, 1958). The change in slope indicates that a different step in catalysis has become rate limiting (Segel, 1975). In the case of Calcutta-hom. the change may denote the onset of protein denaturation. The Ea for Calcutta was determined from the portion of the plot from 20°-30°C. The discontinuity in the Calcutta-homozygote Arrhenius plot suggests instability at the higher temperatures. A range of temperature from 16°C to 44°C by 7° intervals was chosen for the Arrhenius determinations of the purified LD_1's. Both plots were linear throughout the range (Figure 4.6), which means that the variant in heterozygote form behaves similarly to normal LD_1. An Arrhenius discontinuity therefore seems to be inherent in the homozygote alone.

4.2 Thermal inactivation - protein denaturation plots

The second category of thermal energy effect relates to protein stability. The native enzymically active form of most enzymes is converted to a "denatured" form with vastly decreased enzyme activity in aqueous solution at temperatures

exceeding 50°C. Kauzmann (1959) defined protein denaturation as "a process or sequence of processes in which the spatial arrangements of the polypeptide chains within the molecule is changed from that typical of the native protein to a more disordered arrangement". Denaturation does not involve any alteration of the primary amino-acid sequence (Tanford, 1968), but a considerable alteration of the protein's structural organization results, which is characterized by a very large and positive change in entropy. The subunits of a polymeric protein interact with one another by the same kinds of forces that attract one part of the polypeptide chain to another part of the same chain within the subunit (Tanford, 1968). Intersubunit attraction involves, therefore hydrogen bonds, ionic linkages and van der Waals interactions. Edelhoch and Osborne (1976) state that the low temperatures available in aqueous solutions are sometimes adequate to dissociate subunit proteins but rarely capable of unfolding them owing to the stronger interactions that occur within the subunits compared to those between subunits. The short range, non covalent interactions mentioned above, summed over the limited area of contact between the spheroidal subunits of polymeric proteins such as LDH, suggest that the loss of biological activity of the enzyme on heating might be explained by thermal dissociation of the protein into subunits.

The mechanism of heat inactivation of LDH using pig LD_3 as a model was investigated by Sudi (1970). Two models were tested. In the "all or none pathway" the primary steps of inactivation lead to the collapse of the whole tetramer, while

in the "one by one pathway" the primary denaturation event occurs in one subunit. Following the separation of the inactivated subunit the three intact subunits recombine through a number of steps to reconstitute another active tetramer. In most vertebrate species including man, the heart LD_1 homotetramer is more heat stable than the LD_5 homotetramer (see Figure 4.8.a) while hybrid molecules possess intermediate thermostability (Fondy et al., 1964; Okabe et al., 1968; Wieland and Pfleiderer, 1963). In solutions containing heteropolymer isoenzymes the "one by one pathway" predicts that new isoenzymes enriched in the more heat-stable subunit will occur during thermal denaturation. If denaturation occurs by the "all or nothing pathway" then only unchanged tetramers will be recovered from a partially inactivated sample. Under most of the conditions used in Sudi's experiments, the "all or nothing pathway" held true. Only at high protein concentration (1-10 mg/ml) and slow inactivation rates (44-52^{o}C) did enrichment of the more heat stable isoenzymes occur.

The thermal denaturation of a number of vertebrate LDH's have been studied by different workers (Wachsmuth and Pfleiderer, 1963; Fondy et al., 1964; Okabe et al., 1968; Tuengler and Pfleiderer, 1977). Neutral or slightly alkaline phosphate or tris buffers, 50-100 mM were used. Some workers have added BSA to prevent "non-specific" dilution denaturation of purified enzyme solutions, e.g. Levi and Kaplan (1971) diluted LDH in 10 mg/ml BSA. The published denaturation curves of activity plotted against time were generally hyperbolic, dependent on the temperature and therefore the inactivation rate.

4.2.1 Experimental

Amino-acid substitutions frequently result in lowered thermal stability of the variant enzyme (Chapter 1, p.15). To compare the thermal stability of the Calcutta mutant with normal enzyme a number of denaturation experiments were carried out.

1. Starch gel method after McAlpine et al.,(1970)

Samples of variant and normal enzyme, matched for activity, each of 10 µl in 3MM paper wicks were arranged pairwise, normal and variant, across a starch gel and subjected to electrophoresis. The gel was sliced longitudinally into strips each containing one pair of samples. These strips were wrapped in plastic film to prevent dehydration then carefully sandwiched between metal plates, through which water at the required temperature (70°C) was circulated. The strips were removed at set times and chilled to 4°C. The original gel was reconstituted serially, together with an unheated control strip and stained by the tetrazolium method for LDH activity.

2. Aliquot method after Osborne and Tashian (1974).

The enzyme (0.6-0.7 ml) in assay buffer, but containing 2 mM DTT was incubated in stoppered glass tubes in a water bath at the appropriate temperature. At predetermined intervals an aliquot of 75 µl was removed to prechilled tubes. A 10 µl portion of each aliquot was applied to paper wicks for starch gel electrophoresis. The remaining sample was used for the repeated determination of remaining LDH activity by spectrophotometric assay.

The lower intrinsic stability of the A subunit isoenzymes of normal human LDH at 70°C is demonstrated by the starch gel method in Figure 4.8.a. The LD_5 and LD_4 isoenzymes are rapidly inactivated, while LD_3 and LD_2 have been inactivated after 12 minutes under the experimental conditions used. The LD_1 activity, although declining, persists to 40 min. at 70°C.

The comparison of the LDH isoenzymes found in normal haemolysate with those found in Calcutta-1 homozygote haemolysate is made in Figure 4.8.b using the aliquot method. This figure should be compared with Figure 4.10 which plots the spectrophotometrically determined LDH activity of the same aliquot series. The rapid loss of Calcutta activity is observed.

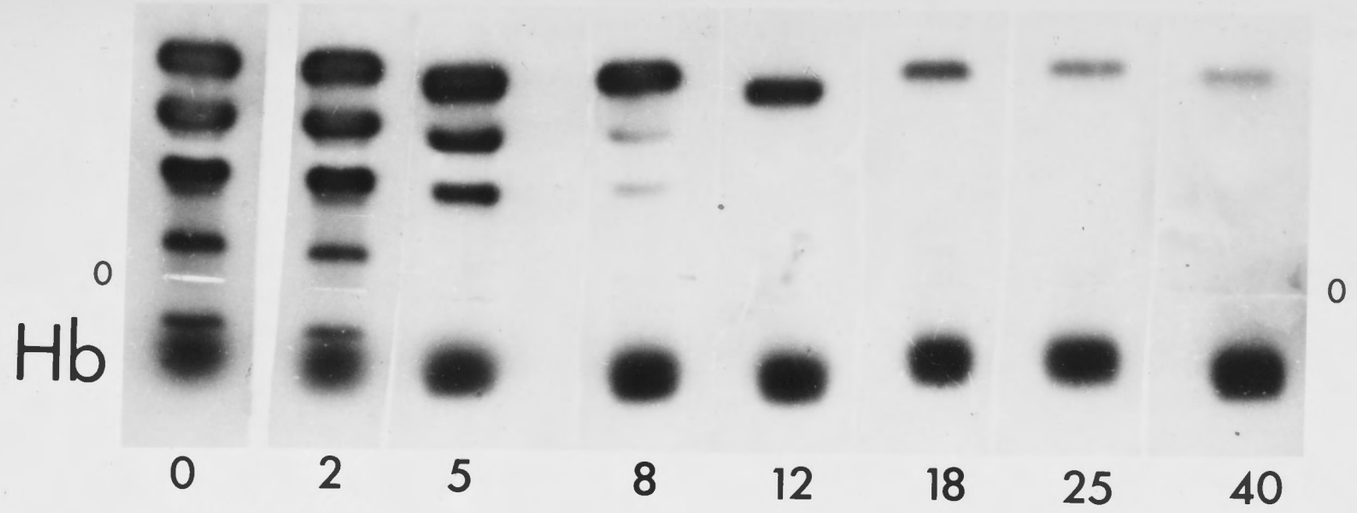
Activity less than can be demonstrated on the starch gel is still detectable kinetically after 2 min. at 70°C (see Figure 4.10). It is evident that Calcutta homozygote LD_1 is less stable than normal LD_1 or even normal LD_2 which persists after 10 min. at 70°C, even though this isoenzyme contains an intrinsically less stable A subunit.

Contrasted with the marked instability of Calcutta homozygote LDH, Figure 4.9.a demonstrates that Calcutta heterozygote isoenzymes appear to be just as thermostable as their normal counterparts at 65°C. No difference in the relative intensities of Calcutta heterozygote LD_1 and normal LD_1 was seen on the gel although a relative falling off of intensity is suggested in the LD_2 and LD_3 positions. Some

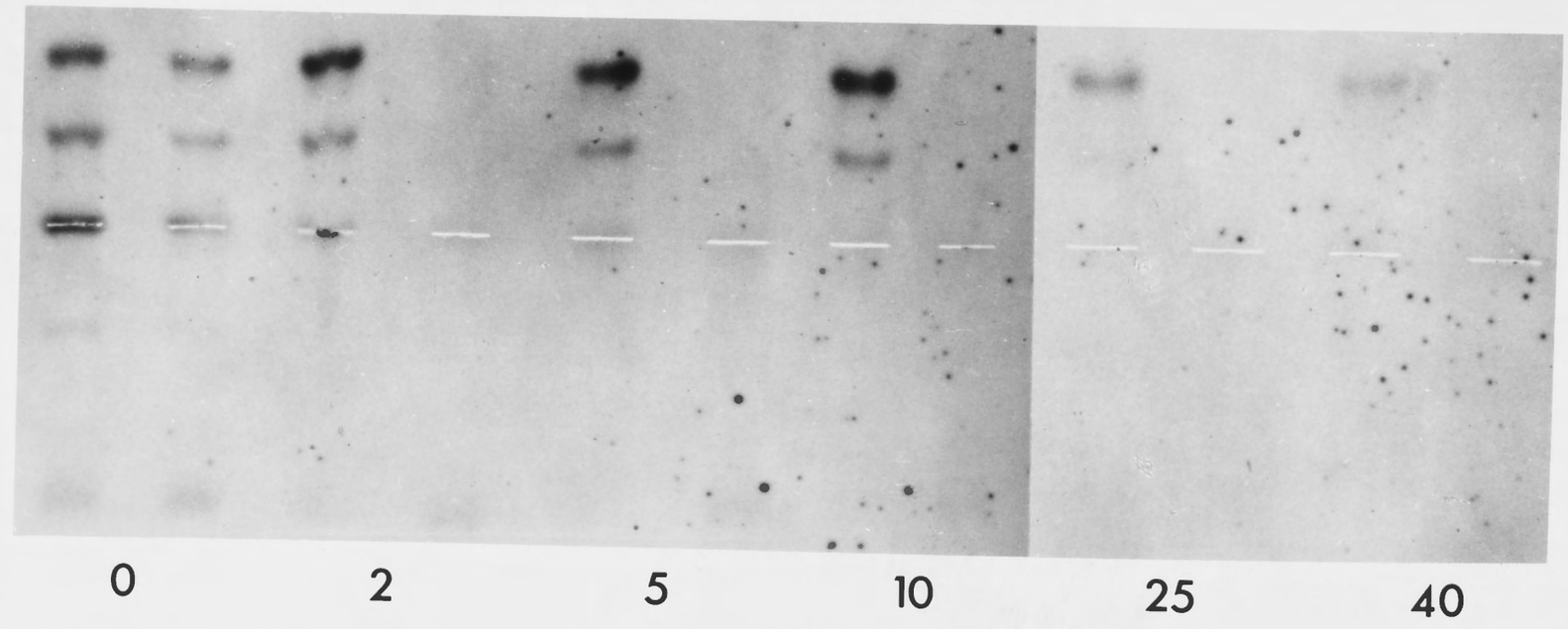
Figure 4.8 Heat denaturation of homozygous haemolysates. Starch gels, pH 7.0, stained for enzyme, anode at the top. (O) origin, Hb haemoglobin.

a. Normal haemolysate, exposed to 70°C for the times indicated (mins) by McAlpine et al., (1970) method.

b. From left, normal (TPC-2254) and Calcutta-homozygote alternately (pairwise), exposed to 70°C for the times indicated (mins) by Osborne and Tashian, (1974) method.



a

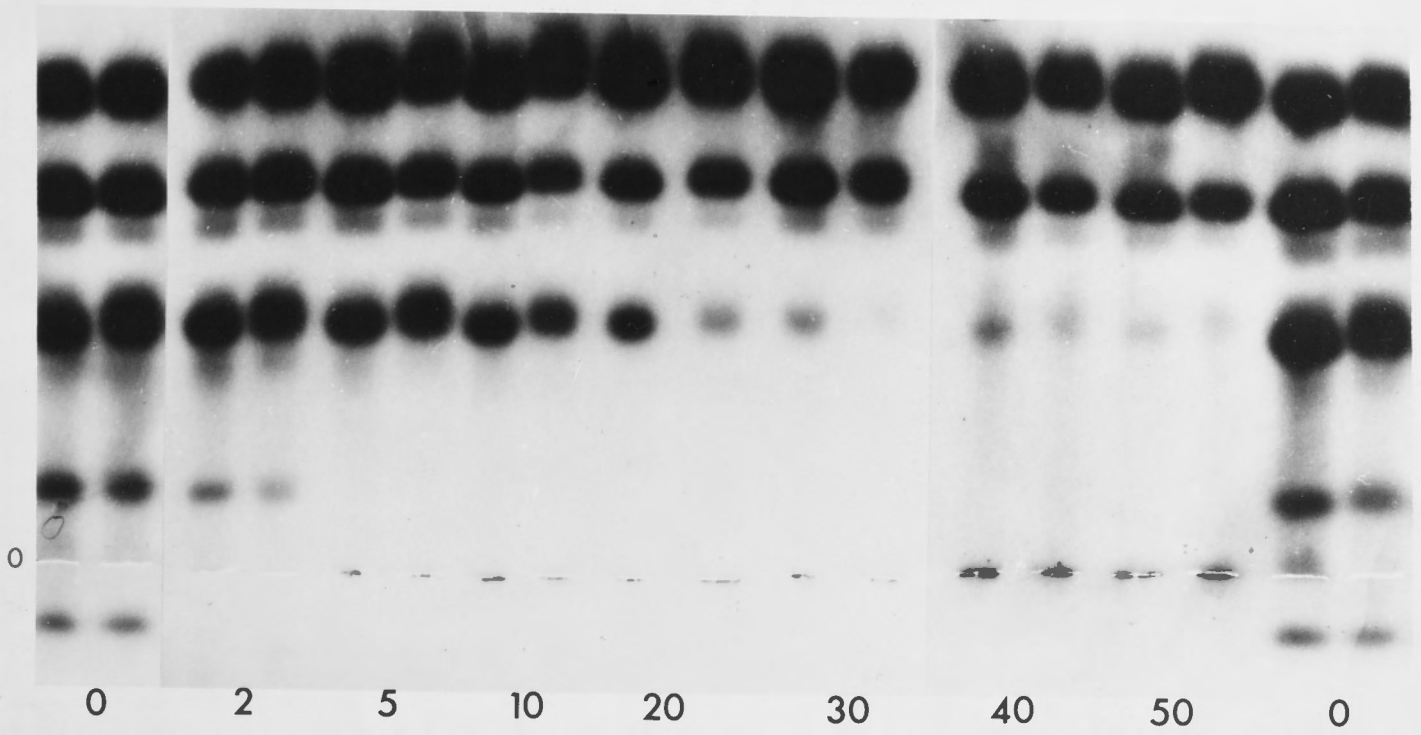


b

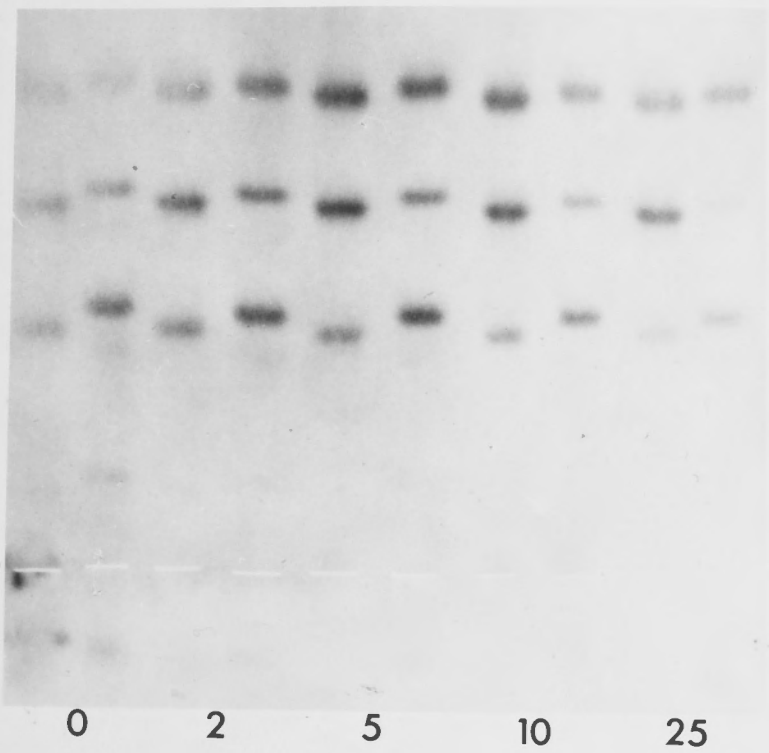
Figure 4.9 Heat denaturation of heterozygous haemolysates. Starch gels, pH 7.0, stained for enzyme, anode at the top. (O) origin, Hb haemoglobin.

a. Normal haemolysate (TPC-2254) and Calcutta-heterozygote (TPC-2245) alternately, pairwise from the left, exposed to 65°C for the times indicated (mins) by Osborne and Tashian, (1974) method.

b. From left, normal (TPC-2254) and Calcutta homozygote alternately pairwise, exposed to 60°C for times indicated (mins) by Osborne and Tashian (1974) method. Note depth of staining deficient toward upper left hand side for technical reasons.



a



b

fast components of the respective Calcutta bands persist after 40 min. Total Calcutta-heterozygote at 70°C shows (Figure 4.10) a coincident denaturation curve with normal.

Denaturation of Calcutta homozygote and normal haemolysates is shown in Figures 4.8.b and 4.9.b. Under the less stringent conditions of 60°C, Calcutta activity survives to 25 minutes. Normal LD_2 activity does not appear to be declining during the time course of the series (Figure 4.9.b) but Calcutta LD_2, equal to normal LD_2 activity at 2 min., becomes relatively less after 25 minutes. Calcutta LD_3 activity, slightly greater at times 2,5,10 min. has approached equal intensity to the normal LD_3 band at 25 min., while LD_1 activities of variant and normal appear to decline to the same extent. Total Calcutta-homozygote activity at 60°C is nevertheless declining relative to normal (Figure 4.10).

The denaturation curves, determined by spectrophotometric assay of activity, plotted against time were hyperbolic - see Figures 4.10, 4.11, as was found by other workers e.g. Fondy et al. (1964). Purified LD_5's from Calcutta and normal (Figure 4.11) showed similar denaturation rates, as did the respective LD_1 isoenzymes (Figure 4.12) Fleisher (1957) studying lobster haemocyanin found that the thermal denaturation of this protein followed first order kinetics. His plots of log [protein] versus time yielded straight lines giving a simple first order rate constant of denaturation. When the data obtained in the current work for loss of activity

with time were treated in this way, the plots of log [residual activity]/time remained hyperbolic. It was concluded that the denaturation process of LDH under these experimental conditions was of higher than first order and not necessarily an order of integral value. No attempt to extract rate constants in order to compare activation energies of denaturation was therefore considered.

Figure 4.10 Plots of heat denaturation of haemolysates at 60°C at 70°C. These plots relate to Figure 4.8.b and Figure 4.9.a,b.

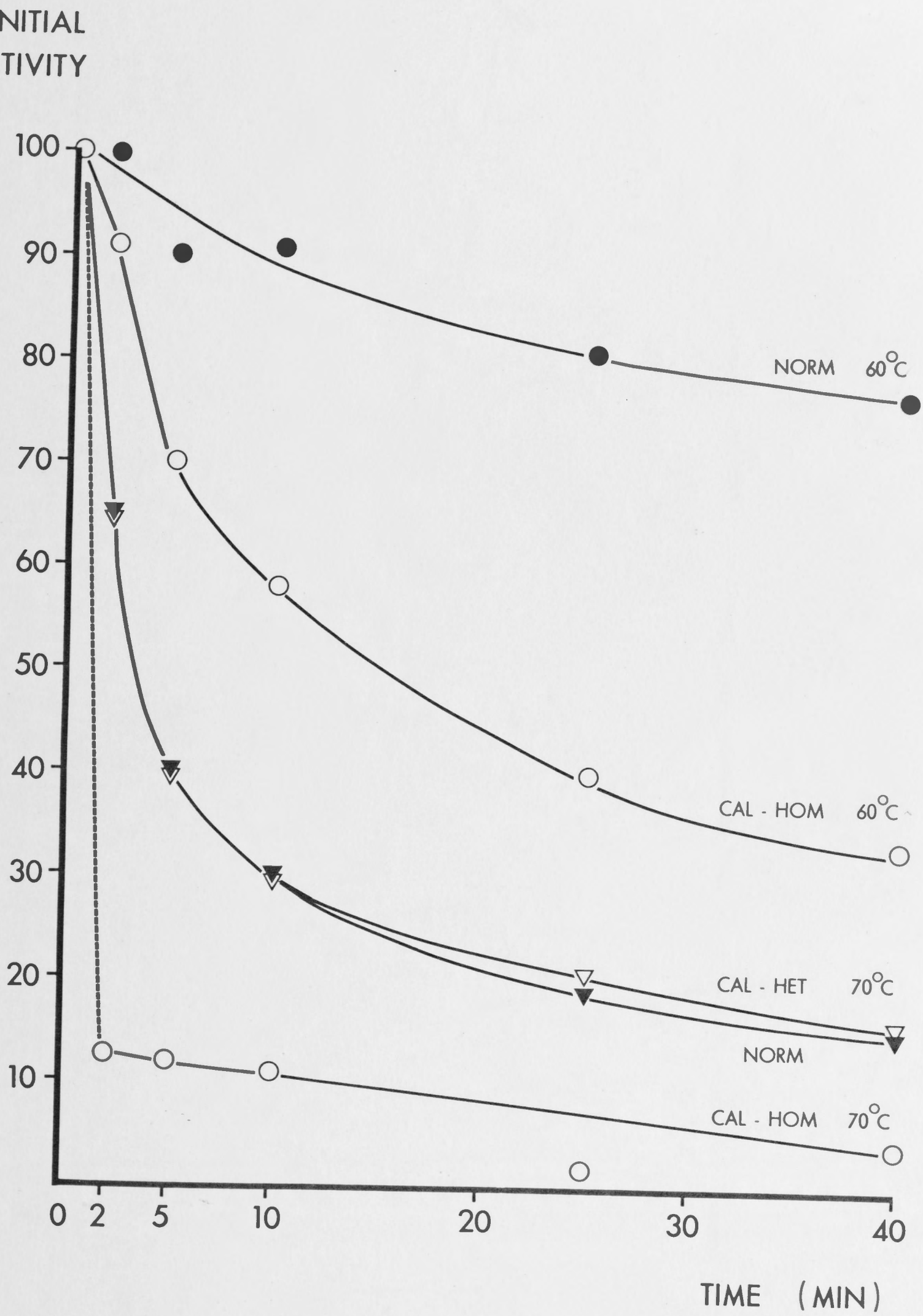

% INITIAL ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
2
5
10
20
30
40
NORM 60°C
CAL - HOM 60°C
CAL - HET 70°C
NORM
CAL - HOM 70°C
TIME (MIN)

Figure 4.11 Plots of heat denaturation of purified LD_5 at 50°C. Variation bars indicate range of velocity determinations.

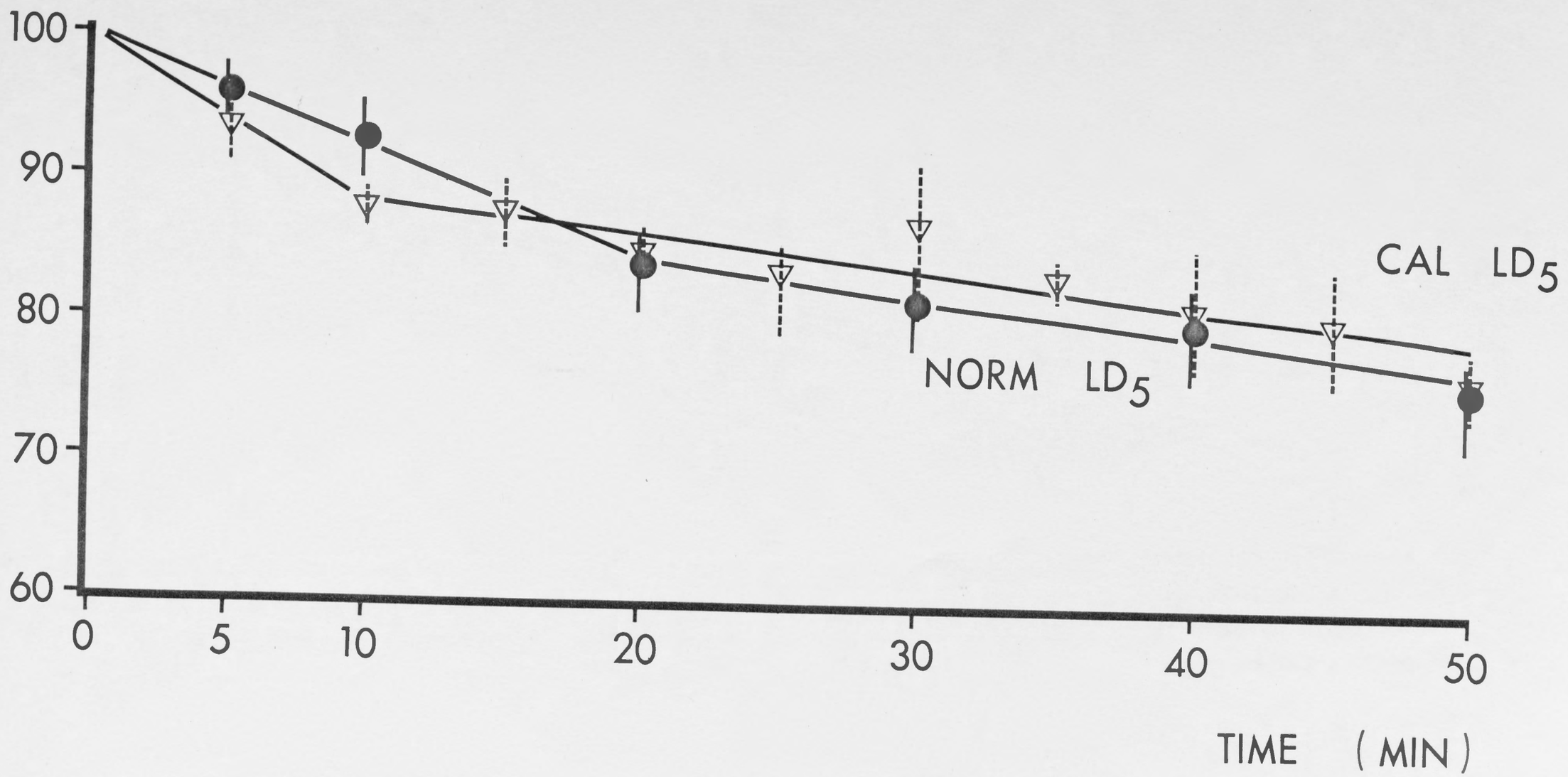
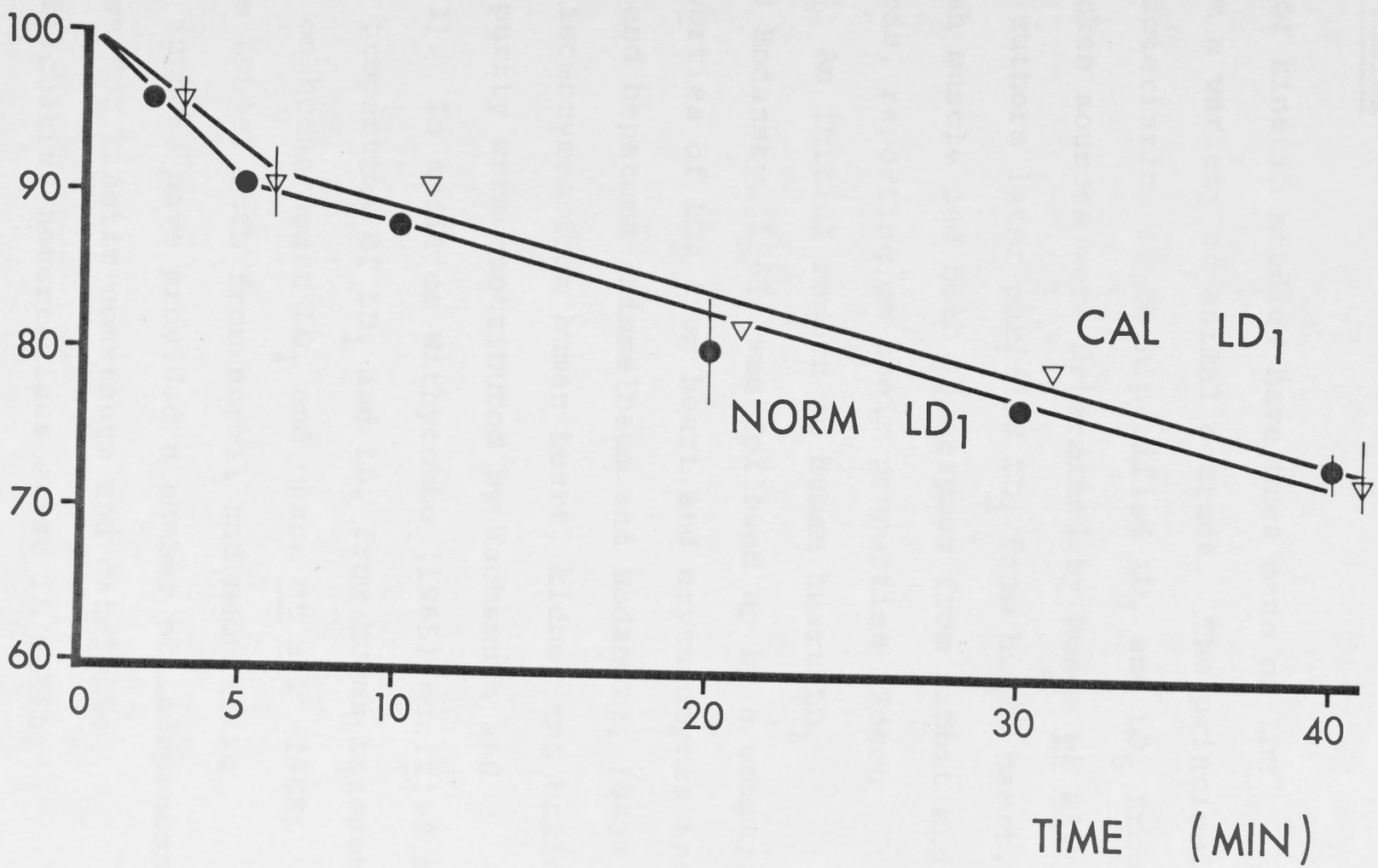

% INITIAL
ACTIVITY
100
90
80
70
60
0
5
10
20
30
40
50
CAL LD5
NORM LD5
TIME (MIN)

Figure 4.12 Heat denaturation of purified LD_1, at 70°C. Variation bars indicate range of velocity determinations. Bars omitted indicate variation did not exceed the area covered by the data point symbol.


% INITIAL
ACTIVITY
100
90
80
70
60
0
5
10
20
30
40
CAL LD1
NORM LD1
TIME (MIN)

5. KINETIC PROPERTIES OF CALCUTTA AND NORMAL LDH

5.1 Earlier studies

A number of kinetic studies have been made on LDH isoenzymes from a variety of animal sources. The principal catalytic characteristics of both purified LD_1 and LD_5 from bovine and chicken sources were determined by Pesce et al. (1964). These authors later purified LD_1 from human heart, LD_5 from dogfish muscle and both isoenzymes from rabbit and a number of birds, reporting on their properties (Pesce et al., 1967). An initial report on human heart LD_1 (Nisselbaum and Bodansky, 1961) was followed up by a comparison of the properties of LD_1 from heart and erythrocytes and LD_5 from liver and hepatoma (Nisselbaum and Bodansky, 1963). Studies on LDH isoenzymes from human heart, kidney and brain, at crystalline purity were contributed by Wachsmuth and Pfleiderer (1963). In addition Withycombe (1965) purified and reported on the properties of LD_1 and LD_5 from human tissues, Balinsky (1966) on human heart LD_1 and Okabe et al. (1968) on human uterine LDH's, both from normal and neoplastic tissues. These authors have provided a number of independently determined values for kinetic constants and catalytic properties. Difficulties nevertheless exist in making comparisons of these values because of the differing assay conditions used - buffers, concentrations, pH's and especially assay temperature, see Tables 5.1, 5.2.

5.2 The comparison of normal and Calcutta kinetics

In the present work, the kinetic properties of normal and Calcutta LDH's were determined under conditions approximating those used by Nisselbaum and Bodansky (1963) except that an assay temperature of 30°C was used. The Michaelis constant for pyruvate and the pyruvate optimum of each enzyme were determined and pyruvate inhibition was examined at various temperatures. The behaviour of each enzyme in response to altered pH was also studied and in each case a pH optimum was estimated.

5.2.1 Kinetic assay methods

The LDH activity in this work was determined spectrophotometrically by following the extinction of NADH absorption at 340 n.m. in the presence of pyruvate as substrate according to the reaction ...

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{lactate} + \text{NAD}^+$$

Assays were performed in a Gilford 2000 recording spectrophotometer coupled to a Beckman DU monochromater. The assay temperature was maintained at 30°C by the circulation of water from a thermostatically controlled water-bath through the jacketted cuvette chamber. The standard assay solution consisted of ...

135 µ moles	(NaH) PO_4, pH 7.4	(0.050 M)
1 µ mole	Na. pyruvate	(0.333mM)
0.25 µ mole	Na. NADH	(0.083mM)

in a final volume of 3 ml.

For assay during the purification stages (column effluents, etc.) the reaction was initiated by the addition of sufficient enzyme (1 µl - 20 µl) to cause an OD_{340} change of between 0.05 and 0.25 O.D. per minute and the extinction curve was recorded for 1-3 minutes. For the kinetic determinations, a constant volume, usually 5 µl of a suitable enzyme dilution was used to initiate each assay. As was noted by Pesce et al. (1967), the purified enzyme in the absence of bovine serum albumin in dilutions suitable for kinetic determinations resulted in declining activity over 30 minutes. The procedure adopted was for the high specific activity purified enzymes under comparison to be diluted into 1 mg/ml BSA in assay buffer and the activities matched (using the standard assay) by small additions of enzyme as required. To ensure that the reaction velocity was dependent on the amount of enzyme added (i.e. $v \alpha [E]$), the amount of enzyme initiating the reaction was doubled, keeping [pyruvate] constant and the new velocity was compared with the velocity obtained with the original concentration. The new velocity had doubled as was expected.

A stock substrate solution was made by dissolving sodium pyruvate in assay buffer, 50 mM $(NaH)PO_4$, pH 7.40, 10 mM in pyruvate and frozen in aliquots until required. The standard assay included 0.1 ml of this solution. For the determination of Km (pyruvate), appropriate dilutions of stock were made to yield final concentrations between 0.033 mM and 2.000 mM

pyruvate. Substrate inhibition studies were carried out between 0.33 mM and 6 mM pyruvate. An NADH concentration, constant at 83.3 µM (0.1 ml of 2.5 mM NADH) was used during these determinations; fresh solution was made up in assay buffer each day. Since the assay is essentially a two substrate reaction it is important that as pyruvate was the varied substrate then [NADH] is saturating, i.e. $[NADH] >> Km_{(NADH)}$. The [NADH] used normally was increased to test its effect on reaction velocity under constant [pyruvate] with LD_1 as catalyst. At 1.62 [NADH], velocity increased by 1.4%; at 1.99 [NADH] velocity increased by 2.1% compared with velocity measured at standard [NADH].

5.3 Determination of Km (pyruvate)

In the standard assay the sodium pyruvate concentration was varied from 0.033 mM-0.333 mM for LD_1 and from 0.333 mM-2.000 mM for LD_5 determinations. Haemolysate was treated as LD_1 for this purpose. The velocity and substrate concentration data obtained were used to estimate the Km (pyruvate) from a Lineweaver-Burk plot graphically. Having obtained a crude estimate of Km, new final concentrations of pyruvate were chosen spanning the Km concentration, at values approximating 0.33-2.00 Km according to the method outlined in Segel (1975). The actual values of substrate concentration were chosen so as to result in evenly spaced reciprocal [s] values so as to avoid the weighting of data in the regression analysis toward the $\frac{1}{v}$ axis, i.e. to higher [s] values. The upper limit of [s] useable was determined by the onset of substrate

inhibition. The experiment was repeated with the new pyruvate concentrations to obtain an accurate determination of the Km (pyruvate) value.

The values obtained at 30°C are tabulated in Tables 5.1 and 5.2 with data of other workers for comparison. The difficulties in comparing results have already been discussed. The results in Figures 4.3 and 4.4 where Km is plotted versus temperature, show that at least a two-fold change in Km can be expected between 23°C and 37°C for human LD_1 considering assay temperature alone. The correlation coefficient r refers to the goodness of fit of the data to the regression of reciprocal velocity on reciprocal [pyruvate] determined by least squares fitting. The Km for pyruvate of Calcutta and normal LD_1 agree (9% variation). The values obtained relate to those of Nisselbaum and Bodansky (1963) who reported 13% difference in Km for presumably normal LD_1 from erythrocytes compared with heart LD_1. The 7° temperature difference probably accounts for their higher result (Table 5.1). In view of the difference obtained between the two normal LD_1's by these authors, no significance can be attached to that between the Calcutta and normal LD_1.

The difference between normal and Calcutta LD_5 Km pyruvate values is 24%. Unlike the results obtained by various authors for LD_1, notwithstanding the difficulties of comparison, those results reported for LD_5 vary from 0.13 mM to 0.83 mM. Errors may have resulted from the use of sub-optimal conditions, for LD_5 especially [pyr] and pH - see Tables 5.2 and 5.3. Determined in this way, Km pyruvate for LD_5 is inherently less precise, and the difference observed

Table 5.1 KINETIC CONSTANTS, HUMAN LD_1

Enzyme	Buffer phosphate (M)	pH	Temp. °C	[NADH] µM	Km(pyr) mM	r	pyr.opt. mM	k_p min^{-1}	Authors
LD_1 heart	0.067	7.4	37	85	0.118±.012		-	78,000	Nisselbaum & Bodansky (1961)
" "	0.067	7.4	37	85	0.068		-	-	" " (1963)
" erythro-cyte	0.067	7.4	37	85	0.059		-	-	" " "
LD_1 heart	0.067	7.2	25	200	-		0.3	-	Wachsmuth & Pfleiderer (1963)
LD_1 -	-	7.4	25	116.6	0.077		0.4	-	Withycombe (1965)
LD_1 heart	0.050	7.5	?25	100	0.056		-	-	Balinsky (1966)
" "	0.100	7.5	25	100	-		-	-	Pesce et al. (1967)
LD_1 uterine muscle	0.050	7.4	-	100	0.03		0.25-0.4	-	Okabe et al. (1968)
Haemolysate normal	0.050	7.4	30	83.3	0.070	.9861	0.45	-	This work
Haemolysate Calcutta-hom.	0.050	7.4	30	83.3	0.082	.9717	0.50	-	" "
LD_1 normal	0.050	7.4	30	83.3	0.056	.9964	0.42	68,000	" "
LD_1 Calcutta	0.050	7.4	30	83.3	0.051	.9966	0.48	50,000	" "

Table 5.2 KINETIC CONSTANTS, HUMAN LD_5

Enzyme	Buffer Phosphate (M)	pH	Temp. °C	[NADH] µM	Km(pyr) mM	r	pyr.opt. mM	k_p min^{-1}	Authors
LD_5	0.067	7.4	25	200	-		∿1.5	-	Wachsmuth & Pfleiderer (1963)
LD_5	0.067	7.4	37	85	0.31		-	-	Nisselbaum & Bodanksy (1963)
LD_5	-	7.4	25	116.6	0.83		1.00	-	Withycombe (1965)
LD_5 uterine muscle	0.050	7.4	-	100	0.13		2.00	-	Okabe et al., (1968)
LD_5 normal	0.050	7.4	30	83.3	0.25	.9851	1.32	102,000	This work
LD_5 Calcutta	0.050	7.4	30	83.3	0.19	.9801	1.17	84,000	This work

r = correlation coefficient for the regression line

k_p = turnover number, µmoles NADH.min^{-1} µmole^{-1} E.

for Calcutta and normal LD_5 probably results from experimental error.

Determination of Km NADH

The standard assay was used for the determination of Km NADH, with pyruvate held constant at the optimum concentration for normal LD_1 of 0.33 mM previously determined (Table 5.1). Normal, variant heterozygote and homozygote haemolysate as well as purified LD_1 were tested. Haemolysates had to be used for the purpose of comparison as purified homozygote LD_1 was not available for these measurements. LD_5's were not tested. The concentrations of NADH were calculated from Lineweaver-Burk plots.

The results obtained are tabulated in Table 5.3. The Km of Calcutta heterozygote was 19% higher than normal while the homozygote variant was 50% higher. These differences suggest a lowered affinity of the variant subunit for coenzyme. The result for heterozygote Calcutta being intermediate between those for homozygote normal and homozygote Calcutta is compatible with a heterozygote composition of half normal subunit and half variant subunit. The results for the purified LD_1's parallel those obtained for the respective haemolysates. Pure Calcutta homozygote LD_1 was not available for inclusion in the comparison. Table 5.3 includes a published result of Km NADH for comparison, but it was determined at higher temperature and pyruvate concentration.

Table 5.3

Km NADH

Enzyme	pH	Temp °C	Pyruvate mM	Km NADH uM	Reference
LD_1 normal	7.4	37	1.00	12	Nisselbaum & Bodansky (1961)
LD_1 normal (purified)	7.4	30	0.33	14	This work
Normal haem-olysate	7.4	30	0.33	16	" "
Cal-het LD_1 (purified)	7.4	30	0.33	17	" "
Cal-het. haemolysate	7.4	30	0.33	19	" "
Cal-hom. haemolysate	7.4	30	0.33	24	" "

A comparison of Km NADH of the variant and normal haemolysates showed that the Km of the variants were higher than that of normal. Higher Km means a lower affinity of the enzyme for the coenzyme. Half maximum velocity of the catalysed reaction is achieved at a higher coenzyme concentration. Lower affinity could result from a subtle conformational change in the coenzyme fold of the mutated subunit, resulting in slightly poorer binding of the NAD molecules. The size of NAD (MW = 680) compared with the substrate molecule pyruvate (MW = 89) implies interaction with many more protein side chains. NAD binding as measured by the apparent Km determination should thus be a more sensitive probe of the active site conformation than pyruvate binding. The results obtained indicate that this might well be so.

5.3.1. Data processing

The Lineweaver-Burk plots were solved for Km and V_{max} using a computer program AECMAIN. This program fits s, v, $S.D._{(v)}$ data to a double reciprocal regression line by least squares manipulation, using the linear version of subroutine POLFIT (polynomial fitting). The subroutine also has the capacity to fit data to a curve, describable by a second order polynomial equation of the form ...

$$Ax^2 + Bx + C = y$$

This facility was used to solve for the abscissa value of the point $\frac{dy}{dx} = 0$ and hence provide a value for the pH optimum in the velocity/pH curves, Section 5.5, and the pyruvate optimum in velocity/[pyruvate] curves, Section 5.4. Program AECMAIN and subroutine POLFIT appear in Appendix-3 and some notes on statistical treatment of the data in Appendix-4.

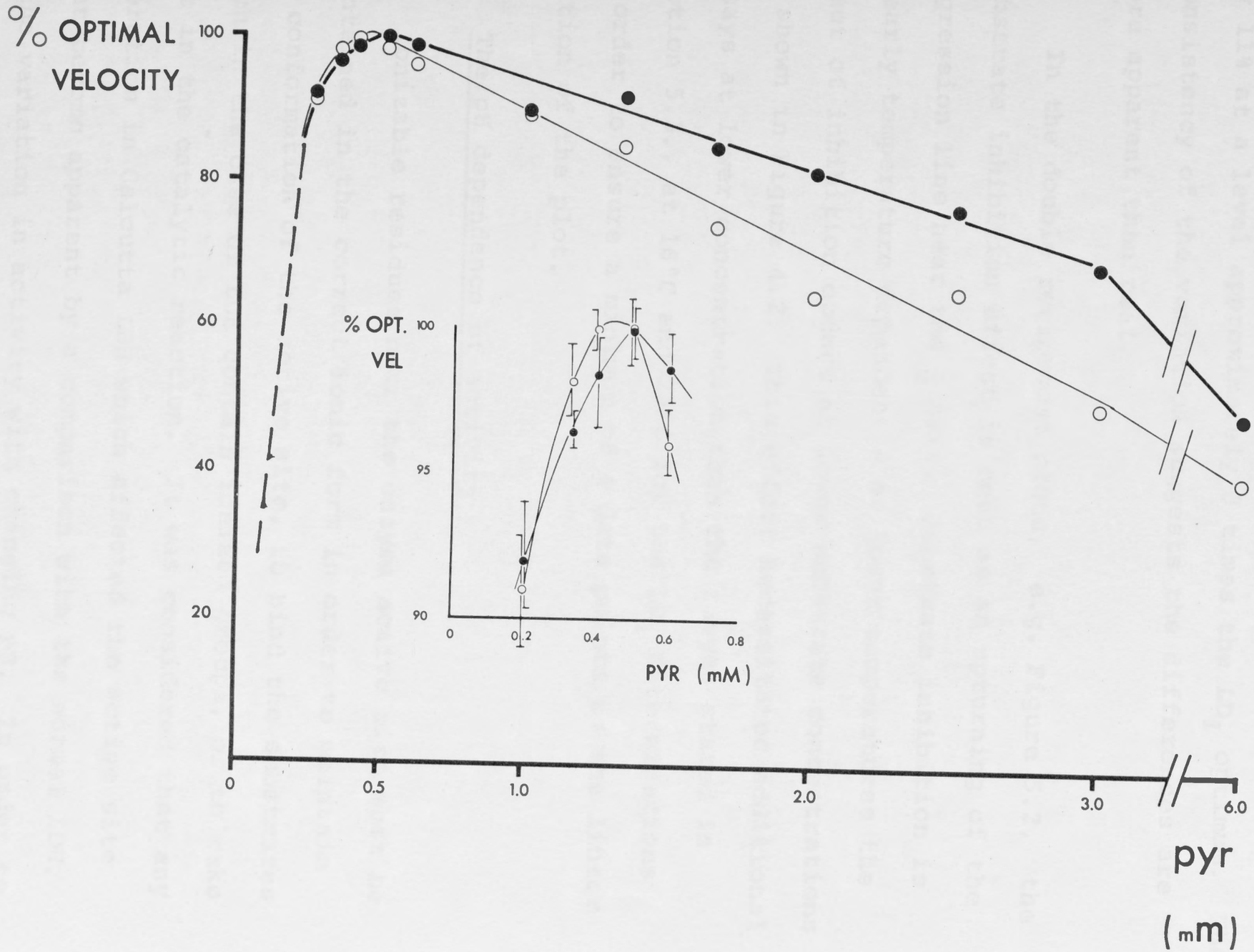
5.4 Substrate inhibition - pyruvate optimum

During the initial determination of Km (pyruvate) of both Calcutta and normal enzymes, the well known substrate inhibition of LDH became apparent. A simple plot of [pyruvate] versus reaction velocity yielded a parabolic curve, e.g. see Figure 5.1. Data near the maximum of this curve was solved for $\frac{dv}{ds} = 0$ to determine the apparent optimum substrate concentration using the polynomial version of POLFIT. Some data on apparent optima are shown in Table 5.1 and 5.2. The

Figure 5.1 % Optimum velocity versus [pyruvate]

solid dots = normal haemolysate.
open dots = Calcutta homozygote haemolysate.

Insert detail in the region of pyruvate optimum.


% OPTIMAL VELOCITY
100
80
60
40
20
0
0.5
1.0
2.0
3.0
6.0
pyr
(mm)
% OPT. VEL
100
95
90
0
0.2
0.4
0.6
0.8
PYR (mM)

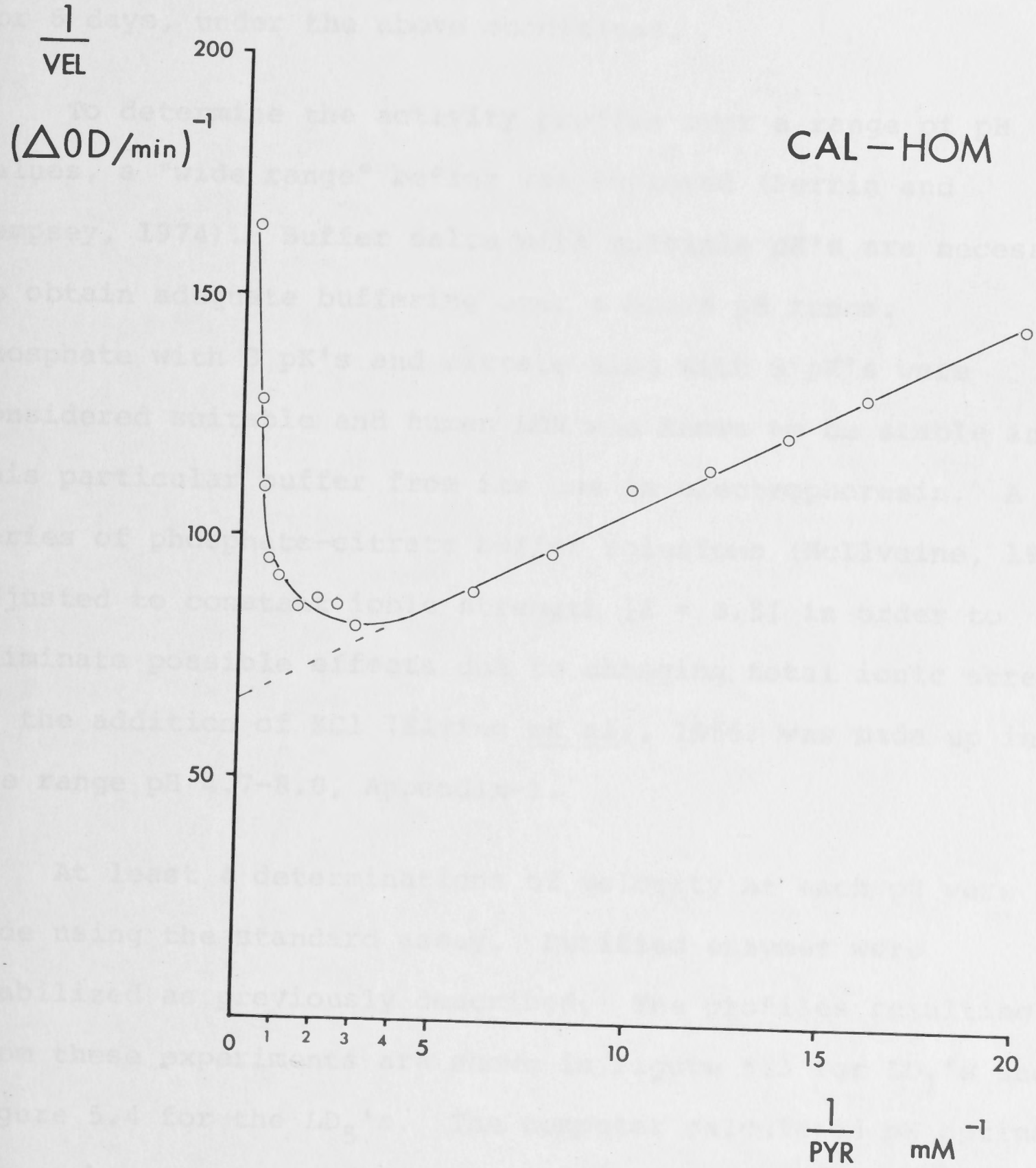
pyruvate optima of LD_1 from normal and Calcutta agree within 12% variation and those of the haemolysates (Table 5.1) agree within 10%. Calcutta and normal LD_5 pyruvate optima differ by 11% at a level approximately 3 times the LD_1 optimum. The consistency of the variation suggests the differences are more apparent than real.

In the double reciprocal plots, e.g. Figure 5.2, the substrate inhibition effect is seen as an upturning of the regression line near the $\frac{1}{v}$ axis. Substrate inhibition is clearly temperature dependent - at lower temperatures the onset of inhibition occurs at lower substrate concentrations as shown in Figure 4.2. This effect necessitated additional assays at lower concentration than the ranges stated in Section 5.3., at 16°C and 23°C for the LD_1 determinations in order to ensure a minimum of 4 data points on the linear portion of the plot.

5.5 The pH dependence of activity

Ionizable residues near the enzyme active site must be maintained in the correct ionic form in order to maintain the conformation of the active site, to bind the substrates which in the case of LDH contain ionized groups, or to take part in the catalytic reaction. It was considered that any alteration in Calcutta LDH which affected the active site might become apparent by a comparison with the normal LDH, of the variation in activity with changing pH. In order to distinguish such a possible change, pH profiles of each enzyme were determined.

Figure 5.2 Lineweaver-Burk plot of Calcutta homozygote haemolysate at 30°C, showing substrate inhibition.


1/VEL
(ΔOD/min)⁻¹
CAL—HOM
200
150
100
50
0
1
2
3
4
5
10
15
20
1/PYR mM⁻¹

Initial stability studies showed that negligible LDH activity was lost from enzyme stored at $4^{o}C$ in phosphate buffers of pH 5.5, 6.5 and 7.5 containing 1 mM DTT provided the protein concentration was 1-5 mg/ml or higher. Dilutions of Calcutta-homozygote and normal haemolysates were stable for 6 days, under the above conditions.

To determine the activity profile over a range of pH values, a "wide range" buffer was employed (Perrin and Dempsey, 1974). Buffer salts with multiple pK's are necessary to obtain adequate buffering over a broad pH range. Phosphate with 3 pK's and citrate also with 3 pK's were considered suitable and human LDH was known to be stable in this particular buffer from its use in electrophoresis. A series of phosphate-citrate buffer solutions (McIlvaine, 1921) adjusted to constant ionic strength [I = 0.5] in order to eliminate possible effects due to changing total ionic strength, by the addition of KCl (Elving et al., 1956) was made up in the range pH 4.7-8.0, Appendix-1.

At least 4 determinations of velocity at each pH were made using the standard assay. Purified enzymes were stabilized as previously described. The profiles resulting from these experiments are shown in Figure 5.3 for LD_1's and Figure 5.4 for the LD_5's. The computer calculated pH optima for each enzyme are tabulated in Table 5.3. It is apparent that the results in each pair of compared enzymes are very similar, haemolysate demonstrating its strong complement of B subunit containing isoenzymes. Segel (1975) lists

Figure 5.3 pH versus activity profile of purified LD_1 at 30°C. Citrate-phosphate buffer I = 0.5. Bars indicate range of variation of determinations.

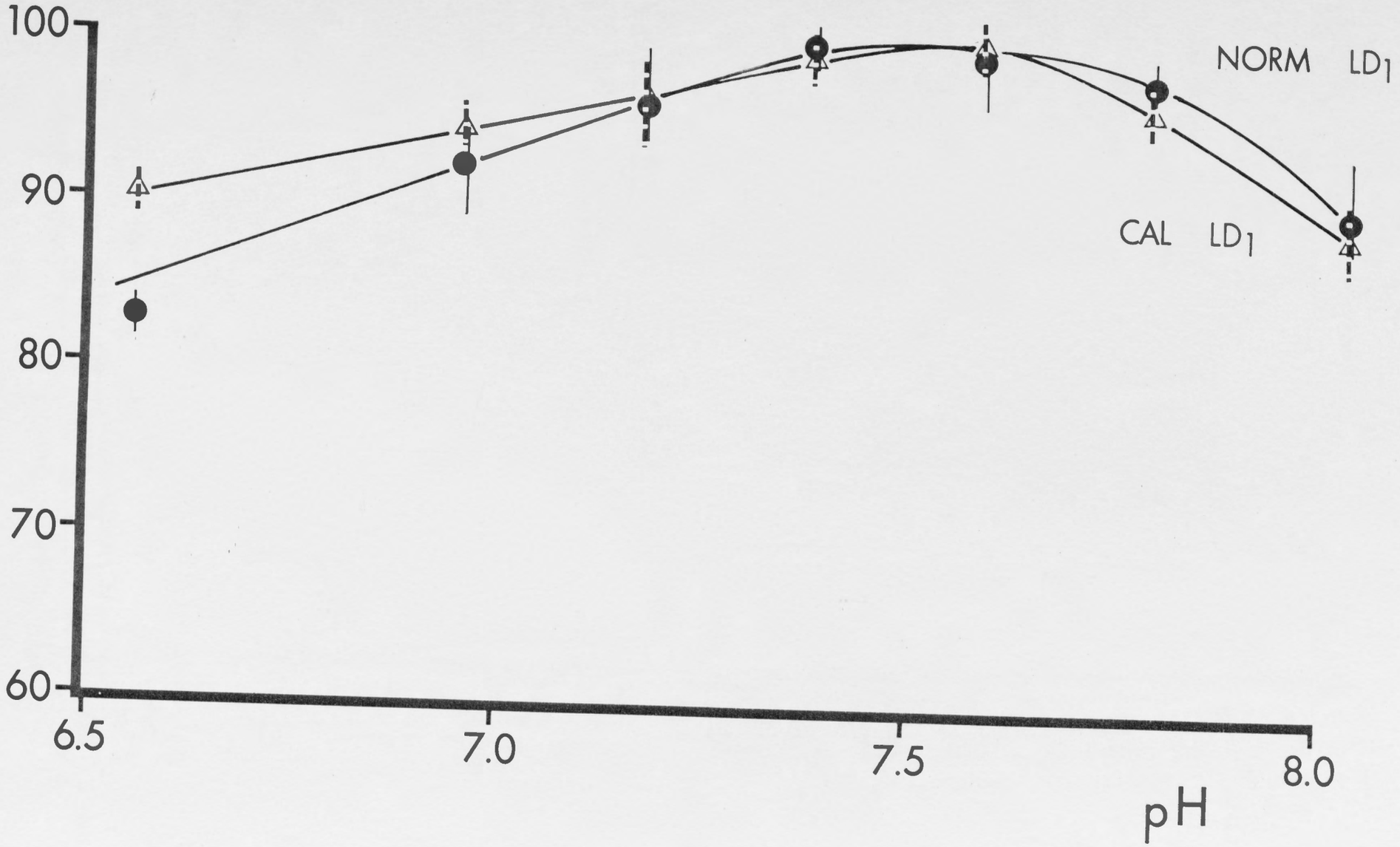
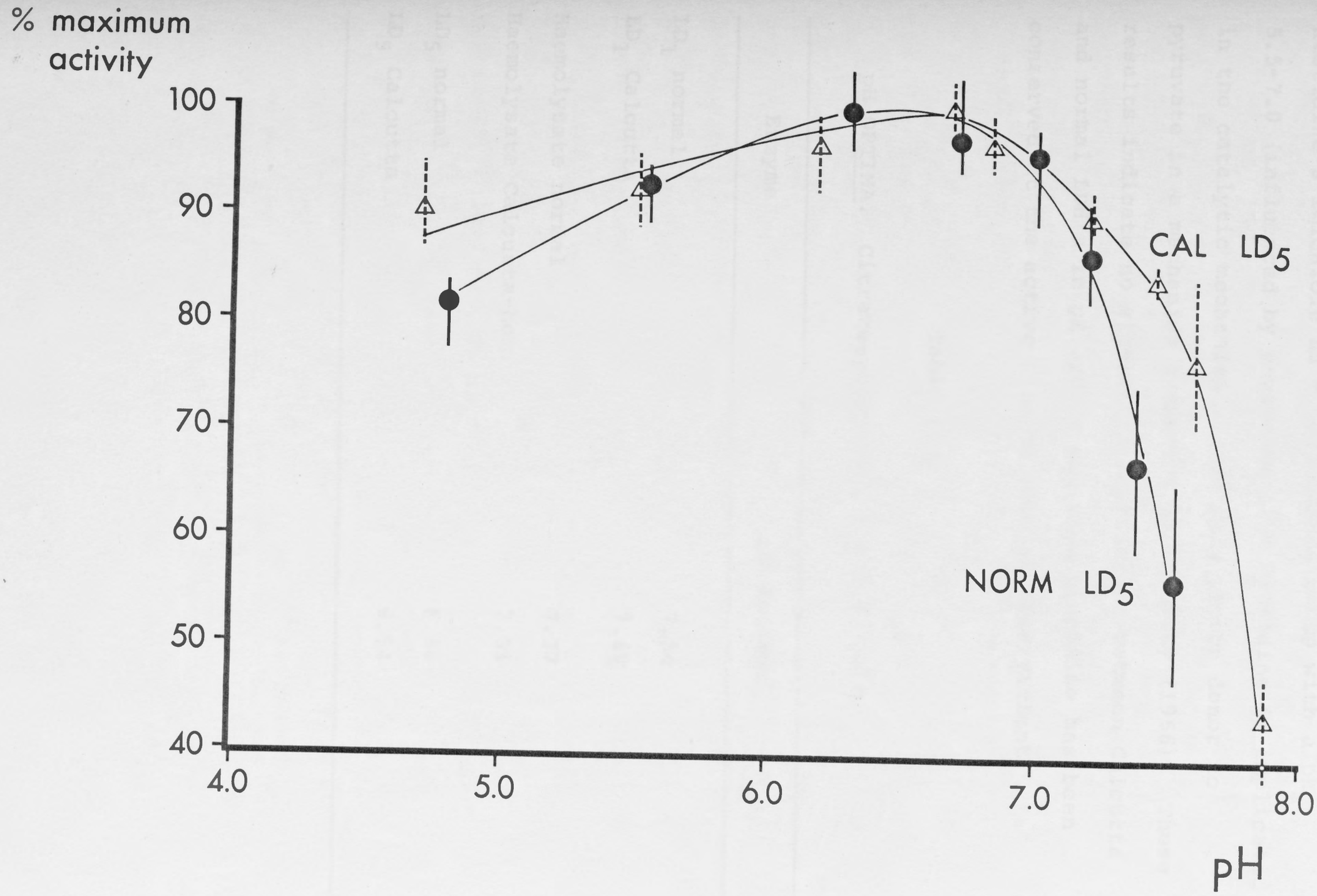

% maximum activity
100
90
80
70
60
6.5
7.0
7.5
8.0
pH
NORM LD1
CAL LD1

Figure 5.4 pH versus activity profile of purified LD_5 at 30°C. Citrate-phosphate buffer I = 0.5. Bars indicate range of variation of determinations.


% maximum activity
100
90
80
70
60
50
40
4.0
5.0
6.0
7.0
8.0
pH
CAL LD5
NORM LD5

histidine's imidazole as a prototropic group with a pk_a 5.5-7.0 (influenced by environment). Histidine is implicated in the catalytic mechanism of LDH as a proton donor to pyruvate in a mechanism suggested by Balinsky (1966). These results indicate no significant difference between Calcutta and normal LDH's in pH optima and that histidine has been conserved at the active site of the Calcutta variant.

Table 5.4

pH OPTIMA. Citrate-phosphate, I = 0.5, 30°C.

Enzyme	pH optimum
LD_1 normal	7.54
LD_1 Calcutta	7.49
Haemolysate normal	7.27
Haemolysate Calcutta-hom.	7.31
LD_5 normal	6.36
LD_5 Calcutta	6.54

6. CIRCULAR DICHROISM STUDY OF LDH STRUCTURE

6.1 Circular dichroism

Two phenomena, circular dichroism (CD) and optical rotatory dispersion (ORD) are observed when a beam of linearly polarized light of a particular wavelength interacts with an optically active molecule such as a protein. The beam may be considered to consist of two components; beams of right and of left circularly polarized light. Their respective electric vectors E_R and E_L travel with different velocities through the molecule, this difference leading to an optical rotation of the line of polarization measured in degrees of rotation, α_λ. Optical rotatory dispersion is the dependence of the rotation on wavelength. An asymetric, optically-active molecule which demonstrates ORD also shows unequal absorption of the left and right circularly polarized light components and the difference in absorbance index ($\varepsilon_L - \varepsilon_R$) is called circular dichroism. The emergent beam is elliptically polarized; the ellipticity θ_λ, being a measure of CD. ORD and CD are closely related phenomena and by using the Kronig-Kramer transform equations, ORD curves can be calculated from CD measurements and vice versa.

6.1.1 Structural determination from models.

Two approaches to the determination of regular structure in a protein molecule have been used, each approach having its advantages and disadvantages. One method has been to use synthetic poly-amino-acids as models of structure and the other has been the use of crystalline proteins whose structure has already been determined by X-ray diffraction methods.

Synthetic polypeptides as models of structure.

Aqueous solutions of several poly-L-amino-acids have been used as models for the estimation of the contributions of each of the structural forms ... α-helix, β-sheet and random form of the polypeptide chain by circular dichroism. Poly-L-glutamic acid and alkaline poly-L-lysine have been used as models of α-helix in protein. Long chain synthetic polypeptides are non-ideal as models of each structure, because of the unique structural arrangement of each native protein such as number and chain-length of the helical segments which are often short, 3-20 peptide units, side-chain effects, and the state of aggregation of subunit proteins.

Models of β structure such as β poly-L-lysine may form intra or inter-molecular β sheets or mixtures of both depending on solvent conditions, whereas in the native proteins quite short sections of sheet are common. Synthetic polypeptides have been seriously questioned as models of the unordered chain in proteins due to the non-agreement of the results determined for polypeptides with those of protein denatured in various ways, e.g. by heat, by acid and by other denaturation methods.

Structure determination using crystalline proteins.

The use of proteins whose structure has been determined by X-ray crystallography e.g. myoglobin and lysozyme has yielded reference CD parameters and curves for α,β and unordered structure. This approach has been used by Chen and Yang (1971). In proteins which were highly ordered in either α-helix (myoglobin) or with β-structure predominating, the estimates of structure from CD agreed within 5% of those determined by X-ray methods (Greenfield and Fasman (1969). For proteins which possessed a low proportion of ordered structure the agreement between the X-ray and the CD data was poorer. X-ray crystallographic data provides information on secondary structure constrained in a close-packed crystalline array whereas with CD on dilute aqueous protein solutions, the constraints on the protein conformation are considerably less rigid. In solution, the protein molecules may be in a number of conformations in dynamic equilibrium. Therefore, different estimates may possibly be obtained for the structure of the same protein in these two different physical states.

The contribution of each form of ordered structure to the CD curve.

The circular dichroism of a protein at any particular wavelength can, as a simplification, be expressed as the algebraic sum of the ellipticities contributed by the three recognised forms of secondary structure: α-helix (H), β sheet (β) and unordered form (R) ...

$$X = f_H X_H^{\bar{n}} + f_\beta X_\beta + f_R X_R$$ Chen and Yang (1971)

X is the mean residue ellipticity [θ]; X_H, X_β, X_R are the reference values for the pure helix, β form and unordered forms, while f_H, f_β and f_R are the respective fractions of each of the three forms in the protein molecule and $\bar{n}$ is the average number of residues per helix.

The CD spectrum in the range of wavelength 190-240 nm (far ultraviolet zone) of a model α-helical polypeptide demonstrates three Gaussian bands at 222, 206, 190 nm, -ve, -ve and +ve respectively (Holzwarth and Doty, 1965). Straus et al. (1969) investigated several globular proteins and found by resolving their CD spectra that essentially the same band locations occurred at 223, 206, 193 nm. The rotational strength of each band for a helix is chain-length dependent, while the environmental effects of amino-acid residues being exposed or buried within the protein will affect the position and magnitude of the three helical bands. The spectrum below 200 nm is characterised by a low signal to noise ratio compared with the spectrum between 200 and 250 nm. In order to measure the strong positive spectral peak due to α-helix at 190 nm, the protein solution may need to be diluted and transferred to a cell of shorter pathlength.

The contribution to the CD spectrum of the residues in the β configuration depends on the number of residues per strand, the number of strands and the polarity of the strands i.e. whether parallel or antiparallel (Woody, 1969).

Computations from the CD spectra of 5 reference proteins (Chen et al. 1974) indicated that the β sheet contributed a -ve peak at 216 nm and weak +ve peaks at 230 and 240 nm. In proteins containing both helices, β-structure and unordered form, the ellipticity of the helices (depending on the wavelength) dominates the ellipticity due to the β form and unordered form.

6.2 Earlier X-ray and CD studies on LDH

Adams et al. (1970) and Rossmann et al. (1971) have shown by high resolution X-ray diffraction analysis of crystalline Dogfish muscle LD_5 that the enzyme possesses considerable regular secondary structure, approximately 45% α-helix and 20-25% β-pleated sheet with 30-35% unordered chain. The conservation of the β-α-β arrangement in the dehydrogenase family of enzymes is now established and has been discussed in Section 1.2.2. The X-ray work of Rossmann's group has shown that the LDH β sheet is made up of 6 strands, the sheet itself being twisted from planar. β strands of both polarity were observed in the LDH subunit. Chen et al. (1974) determined the amount of helix and β form in eight reference proteins (including Dogfish muscle LD5) by analysing their CD spectra. Using a value of 11 for $\bar{n}$, LDH was found to possess 39% helix and 17% β form, slightly lower values than were obtained from the X-ray work.

Levi and Kaplan (1971) have used CD to compare native Dogfish muscle LD_5 with the same enzyme dissociated in 5.3 M lithium chloride, then reassociated in the presence and absence

of NADH. The structure of the LDH reassociated in the presence of NADH was judged from the CD spectrum to be closer to that observed for the native enzyme. Comparative studies by Hamada (1975) of LDH purified from normal human uterus, uterine myoma and from uterine myosarcoma showed that the amount of isoenzyme LD_5 was greater in the neoplastic tissues. It was shown that the LD_5 isoenzyme from these tissues exhibited greater thermostability than the LD_5 from the normal tissue. Hamada's circular dichroism results suggested that the isoenzyme from the neoplastic tissue had substantially less helical structure, and he inferred that the less folded structure was associated with the greater heat stability. The primary structure, molecular weights and immunochemical properties of the various LD_5's were found to be unaltered. In the present work the CD spectra of each of the normal and Calcutta LD_1 and LD_5 isoenzymes were determined. By a comparison of these curves it was considered that possible effects of the Calcutta mutation on the regular structural elements of the LDH protein, or resultant conformational rearrangements, might be detected.

6.3 Experimental

The spectra of the purified human LDH's were recorded in a CARY-60 Spectropolarimeter with a Model 6001 circular dichroism accessory unit.

6.3.1 Calibration of the spectropolarimeter

A recent paper by Tuzimura et al. (1977) showed that

in a quality control survey, deviations occurred in the spectropolarimeters taking part. ~~of~~ The molecular ellipticities of the same α camphor-sulphonic acid solution at 220 nm, deviated ~~of~~ up to > 30%. ~~were reported.~~ Careful calibration is therefore essential for meaningful circular dichroism. The calibration of the CD unit in this work was checked by recording the ORD and CD spectra of a solution of α camphor-sulphonic acid and using the Kronig-Kramers transform to compare these two spectra. The agreement was excellent throughout the entire wavelength range used in this work. The calibration of the CD unit was therefore accepted as reliable. The absence of baseline drift was ensured by recording the spectrum of a nitrogen gas stream both before and after making the protein measurements.

6.3.2 Protein measurements

The purified human LDH's were exhaustively dialysed against 0.1 M sodium phosphate buffer pH 7.0 containing 1 mM DTT (Appendix 6) and the CD spectra were determined between 350 nm and 200 nm. In the far ultraviolet (200-250 nm) the spectra were determined with the protein solutions in a 0.1 cm pathlength cell, while for measurement in the near ultraviolet (250-350 nm) the same solution in a 2.0 cm cell was used. The solvent "background" was determined in the same cells after thoroughly washing with solvent. Ellipticity values of the protein solutions were corrected for the solvent spectrum obtained. The spectrum for a particular protein was recorded twice and occasionally three times over the same tracing in order to average the noise. The recordings were

made at the slowest scan speed. Smooth curves were drawn through the spectral traces and the ellipticity θ, was measured from the scale at 2½ nm intervals, or at more frequent intervals near the peaks and troughs. These results were converted to mean residue ellipticity $[\theta]$ according to the equation ...

$$[\theta] = \theta \left(\frac{M.R.W.}{10.l.c'}\right) \text{deg.cm}^2\text{.dmole}^{-1}$$

where l is the cell path-length in cm, c′ is the protein concentration in g/cm^3, θ is the measured ellipticity taken from the trace at a particular wavelength. A value for the mean residue weight (M.R.W.) of 105.74 was used for both LD_1 and LD_5, assuming a subunit molecular weight of 35,000 and 331 residues/subunit (Taylor et al., 1973).

Protein concentration was determined at the completion of each spectrum by measuring the absorption at 280 nm and applying the absorbance index at 280 nm of $A^{1\%}_{1\ cm} = 12.6$. Correction for light scattering was made by subtracting the value $A_{350} \left(\frac{350}{280}\right)^{2.5}$ from each A_{280} measurement. Protein concentrations used were in the range 1.5 - 16.2 $\times 10^{-6}$M. The spectra of the normal and Calcutta LD_1's are presented in Figure 6.1 and those of the normal and Calcutta LD_5's in Figure 6.2. Data points between 300 nm and 350 nm have been omitted from the plots since zero ellipticity was exhibited in this range.

Figure 6.1 Circular dichroism of LD_1 in far and near ultraviolet. Bars indicate range of variation of the multiple traces. Cary-60 spectropolarimeter.

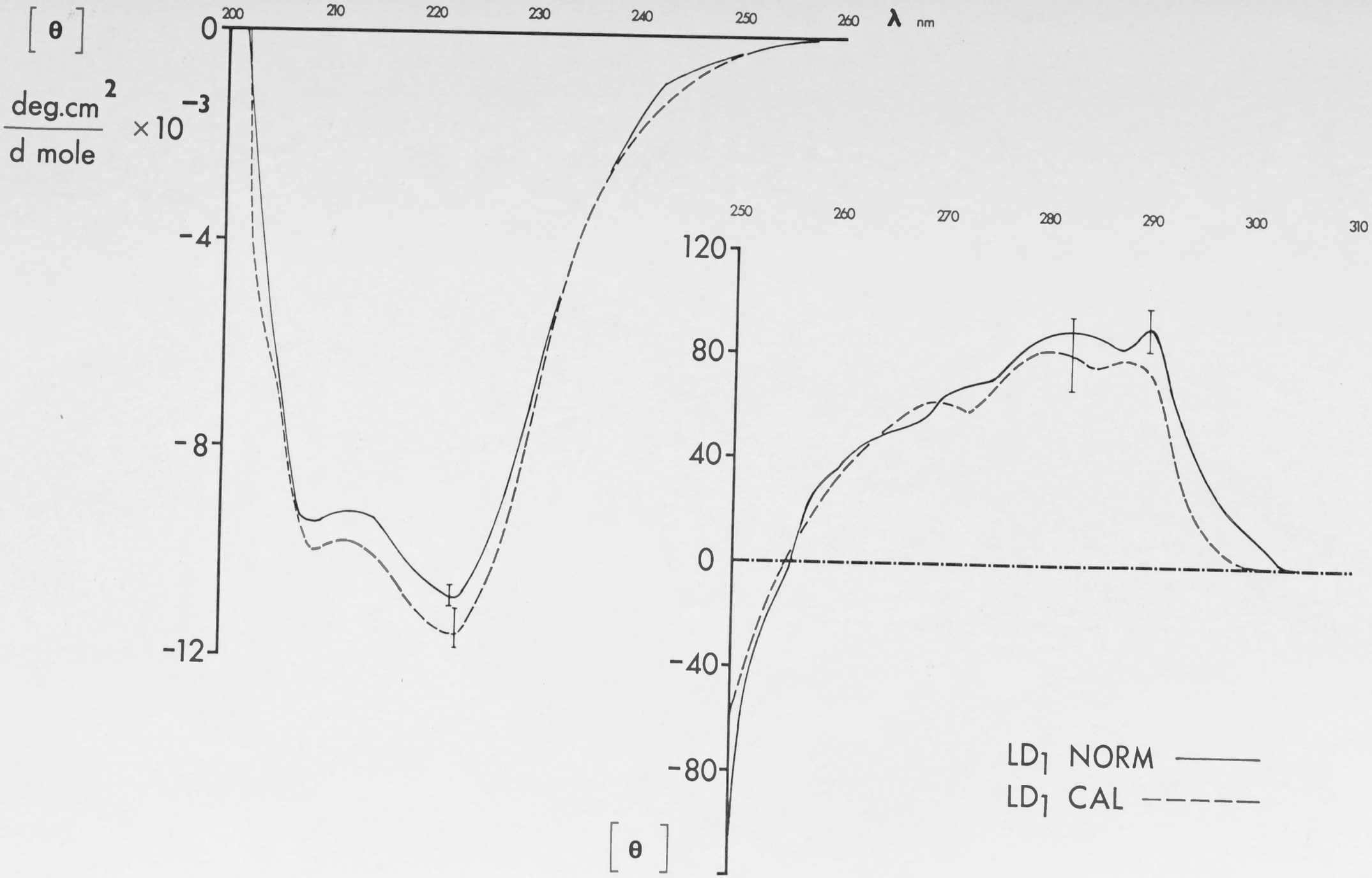
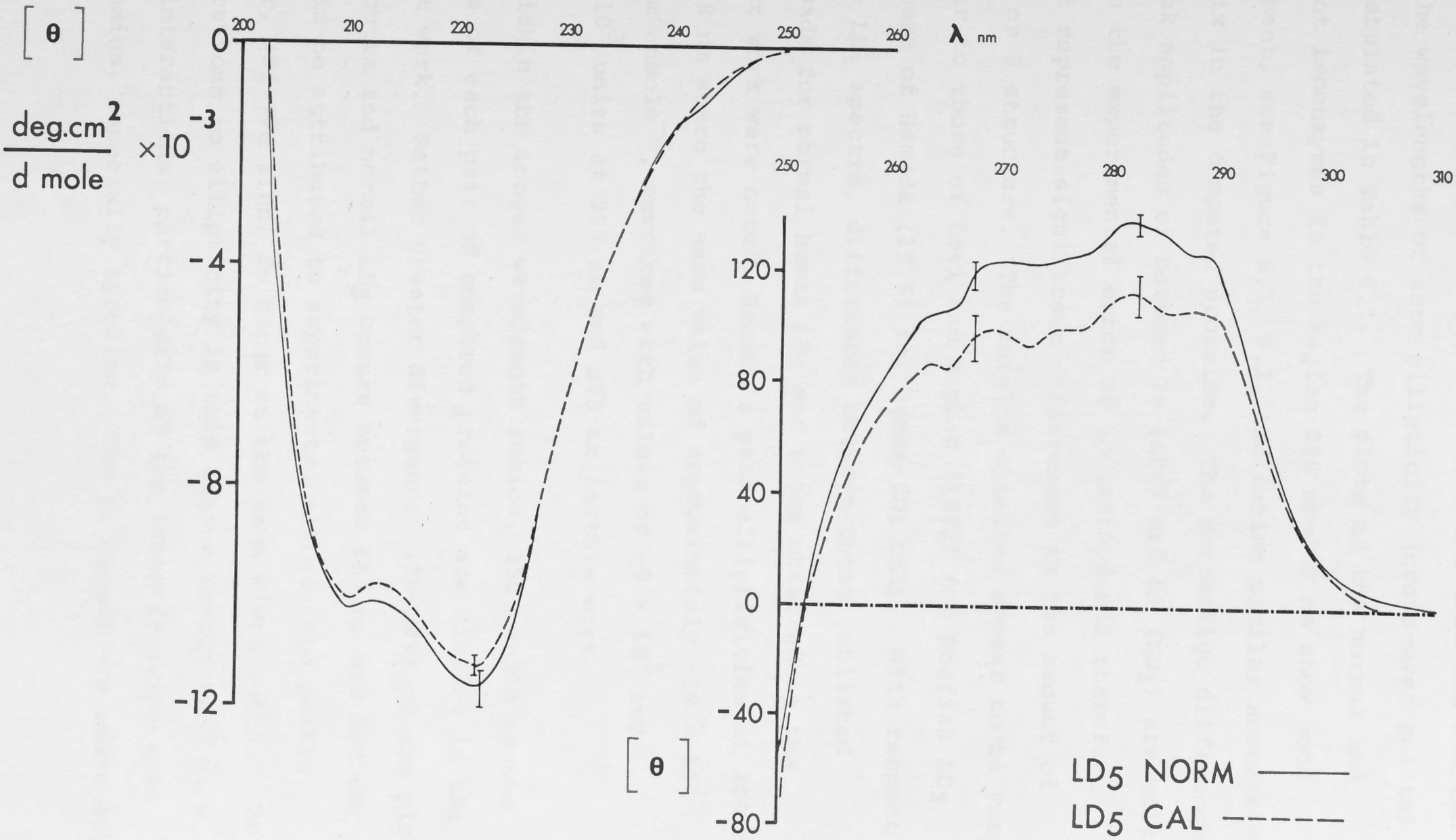

[θ]
deg.cm²/d mole
×10⁻³
0
-4
-8
-12
200
210
220
230
240
250
260
λ nm
250
260
270
280
290
300
310
120
80
40
0
-40
-80
[θ]
LD1 NORM
LD1 CAL

Figure 6.2 Circular dichroism of LD_5 in far and near ultraviolet. Bars indicate range of variation of the multiple traces. Cary-60 spectropolarimeter.


[θ]
deg.cm²/d mole ×10⁻³
0
-4
-8
-12
200
210
220
230
240
250
260
λ nm
250
260
270
280
290
300
310
120
80
40
0
-40
-80
[θ]
LD5 NORM
LD5 CAL

The molar ellipticity values obtained from the extrema and the wavelengths of zero ellipticity (cross-over points) are tabulated in Table 6.1. The plots of the normal and variant isoenzymes in the region 200 nm-250 nm show good agreement, see Figure 6.1, 6.2, indicating similar amounts of α-helix in the compared proteins. The percentage difference in peak amplitudes of between 2% (LD_5) and 6% (LD_1) are well within the experimental error of the method and therefore do not represent significant differences in the amount of helix or β structure. The results obtained appear to be very similar to those of Levi and Kaplan (1971) for Dogfish LD_5 and those of Hamada (1975) for human LD_5 only. With respect to the LD_1 spectra, differences between those published by Hamada for normal human LD_1 and those obtained in the present work were noted. Hamada's peak ellipticities at 209 nm and 218 nm share the same value of approximately -19×10^3 deg. $cm^2 . dmole^{-1}$, compared with values of -9×10^3 and -11×10^3 units at 209 nm and 223 nm in this work.

Within the longer wavelength region, 250 nm-350 nm the spectra of each pair of compared proteins are similar in the current work. Rather greater divergence (15-20%) of the plots of Calcutta and normal LD_5 occurs between 260 nm and 290 nm than can be attributed to experimental error. The peaks however, (of +ve sign) do occur at the same wavelengths. The contributions to ellipticity in this region result from side chain interactions, particularly of the aromatic amino-acid side chains, especially tyrosine. The strongest +ve peaks are

Table 6.1 EXTREMA AND ZERO VALUES OF C.D. SPECTRA FOR HUMAN LDH VARIANTS

Protein	200 nm - 250 nm				250 nm - 300 nm				
	[θ] deg. cm^2. d $mole^{-1} \times 10^{-3}$								
	zero value	209 nm	223 nm	zero value	263 nm	269 nm	282 nm	289 nm	zero value
LD_1 NORMAL	201-202nm	-9.4	-10.8	255 nm		0.063	0.081	0.078	300 nm
LD_1 CALCUTTA	201-202	-10.0	-11.6	255 nm		0.059	0.090	0.090	305 nm
		210 nm	222 nm						
LD_5 NORMAL	201-202	-10.1	-11.6	252-253 nm	0.103	0.123	0.138	0.126	310 nm
LD_5 CALCUTTA	201-202	-9.9	-11.3	252-253 nm	0.083	0.099	0.113	0.107	305 nm
Differences in amplitude between normal and Calcutta LDH variant									
LD_1 NORMAL AND CALCUTTA		6%	6%			6%	10%	13%	
LD_5 NORMAL AND CALCUTTA		2%	3%		19%	20%	18%	15%	

those due to the vibronic transitions of tyrosine at 282 nm and at 288 nm as discussed in Su and Jirgensons (1977), (see both Figure 6.1 and Figure 6.2). A further peak due to tyrosine occurs at 269-270 nm which merges with a +ve peak at 266 nm resulting from the phenylalanines present. Phenylalanine also contributes to positive ellipticity at 258 nm. Differences between human LD_1 and LD_5 proteins are demonstrated by the results obtained in this region. In the LD_1's ellipticity at 258 nm was 21-29 units while in the LD_5's at the same wavelength, 61-74 units. At 266 nm the LD_1 ellipticities were 51-55 units while those of the LD_5's were 90-107 units. The ellipticities may reflect the higher phenylalanine content of LD_5 protein. Tryptophan contributes a +ve peak at 293 nm and a weak negative peak at 304 nm. No -ve peak was observed in either the LD_1 or LD_5 spectra at 304 nm, but Calcutta LD_1 possessed an ellipticity of 34 units at 293 nm while the normal LD_1 was 56 units at this wavelength, suggesting a difference in composition or of conformation of tryptophan residues.

These results obtained are significantly different from those obtained for normal human LD_5 in the same buffer by Hamada. He plotted the principal peak at 260 nm of +80 units, together with a small -ve peak at 285 nm of approximately -15 units, compared with a peak of +138 units at 285 nm in this work. Hamada used protein of concentration 1.5×10^{-7}M for his determinations, while the LD_5's used in this work were 3.1 and 16.2×10^{-6}M.

In summary, comparable circular dichroism spectra were obtained for normal LD_1 and Calcultta LD_1; for normal LD_5 and for Calcutta LD_5. No major conformational alterations in secondary or tertiary structure as a result of the Calcutta mutation, detectable by circular dichroism in the far ultraviolet were suggested by the results. Certainly the gross alterations in both the amount of α-helix and in the "side chain" region found in pathological "conditioned-LDH's" by Hamada (1975) were absent in the Calcutta variant. The near ultraviolet region, 255 nm-300 nm, of the ellipticity plot suggests possible differences, perhaps caused by altered aromatic residue content or in altered conformation between the normal and Calcutta LD_5 proteins.

7. AMINO ACID ANALYSIS AND PEPTIDE MAPPING

Peptide mapping of proteins hydrolysed by specific endoproteases such as trypsin, chymotrypsin or papain, or by residue specific hydrolytic reagents such as cyanogen bromide has provided a valuable visual means of comparing related proteins. The technique has been fundamental to the advances made in the nature of human haemoglobin variants, and some progress has also been made with human enzyme variants such as those of G6PDH and carbonic anhydrase. However the author is not aware of any such similar studies on human LDH variants. The recognition of protein variants by zone electrophoresis almost invariably provides the stimulus for their further investigation by peptide mapping, while the chromatographic separation in the mapping procedure may be capable of resolving previously unsuspected hydrophobic or neutral substitutions.

7.1 LDH peptide mapping

Peptide mapping and amino-acid analysis of animal, including normal human LDH's has been used extensively to highlight the divergence of the homologous LD_1 and LD_5 proteins of a single species (Pesce et al., 1967; Wachsmuth et al., 1964). In addition comparisons of each homotetramer have been made between phylogenetically related animals (Pesce et al., 1967).

Although Hamada (1975) has compared the amino-acid compositions of both human LD_1 and human LD_5 from normal and neoplastic tissues and concluded that the primary sequence is probably unchanged, peptide mapping was not employed.

In the present work, tryptic cleavage and mapping of the resultant peptides from the purified LDH isoenzymes was carried out, in an attempt to elucidate the nature of the Calcutta variant and to identify possible amino-acid substitutions. Purified normal LD_1 was compared with purified Calcutta-1 homozygote LD_1 and the purified normal LD_5 was similarly compared with Calcutta-1 heterozygote LD_5.

7.2 Experimental methods

The purified enzyme (2-5 mg) was dialysed against 2 changes of 50 mM NH_4HCO_3, transferred to a round-bottomed flask, shell frozen in dry ice/ethanol and subjected to lyophilization overnight.

7.2.1 Performic oxidation

Performic acid (0.5 ml 100 vol. H_2O_2, 9.5 ml 98% formic acid) was preformed at room temperature for 1-2 hr and chilled to $4^{o}C$. The lyophilized protein was dissolved in 1 ml 98% (v/v) formic acid, chilled and 3 ml performic acid added. After oxidation for 2-3 hr on ice the sample was re-lyophilized. The flocculent, white, oxidized protein was redissolved in 1 ml formic acid, 10 ml of double glass distilled H_2O was added to decompose excess peroxide and the sample was again lyophilized.

7.2.2 Amino-acid analysis

Approximately 1 mg of freeze-dried protein was weighed out, transferred to a thick-walled, acid-washed glass tube and 2 ml of 6M HCl added. A drop of liquid phenol was added

to protect the tyrosine against oxidative chlorination. The tubes were heat sealed under reduced pressure and the samples hydrolysed at 110°C for 22 hr. The hydrolysate was rotary evaporated, dissolved in pH 2.2 sodium citrate buffer 0.066M (Moore et al., 1958) and analysed in a Beckman 120 C amino-acid analyser. This analyser utilizes two columns. The short column was packed with Beckman Custom Spherical Resin PA-35 and eluted with 0.116M sodium citrate, 0.099M HCl buffer, pH 5.28 to resolve lysine, histidine and arginine. The long column was packed with Beckman Custom Spherical Resin AA-15 and eluted with 0.066M sodium citrate, 0.151M HCl buffer, pH 3.28 followed by 0.066M sodium citrate, 0.101M HCl buffer pH 4.25. The long column resolves the neutral and acidic amino-acids. The buffer flow rate was 60 ml/hr and the temperature 52°C.

7.2.3 Tryptic digestion

The oxidised, lyophilized protein (1-4 mg) was suspended in 5 ml of 0.5% w/v NH_4HCO_3, pH 8.0. Trypsin (1 mg/ml in 0.01M HCl) was added in the proportion 0.01 mg/mg protein and digestion allowed to proceed for 2 hr at 37°C. A second aliquot of trypsin was added (final trypsin concentration was 2% by weight) followed by a further 2 hr at 37°C to maximise hydrolysis. The digest was then shell-frozen and lyophilized overnight to remove the ammonium bicarbonate and water. "Core" (peptides with limited solubility at pH 4) was precipitated if necessary by suspending the tryptic hydrolysate in 2 ml H_2O prior to the dropwise addition of 0.5% NH_4HCO_3 to solubilize the material. Dropwise addition

of glacial acetic acid (20-50 µl) followed, to a final pH of 4 and the precipitated "core" material separated by centrifugation. The precipitate washings were returned to the supernatant which was lyophilized.

7.2.4 Peptide mapping

Peptide maps were prepared by dissolving the soluble peptides in a minimum of water (30µl-50µl) and applying this solution as a 2cm streak to a sheet of Whatman 3MM chromatography paper, using a finely drawn pasteur pipette. The sheet was wetted out with electrophoresis buffer applied evenly to each side of the origin, and blotted to remove excess buffer. High voltage vertical electrophoresis with redistilled mineral turpentine as coolant, for 1½ hr at 40 v/cm (total potential 2000v, for 53 cm paper and 80-150 mA) was carried out. The buffer system was acetic acid:pyridine: water (1:1:38 by volume), pH 4.7. The tanks were designed after Michl (1958). After air drying and viewing under UV light the dried strips were cut out, sewn onto fresh Whatman 3MM paper, the back excised, and subjected to ascending chromatography at right angles to the electrophoretic separation at room temperature for 20 hours. The chromatography buffer was butanol, acetic acid, water, pyridine after Waley and Watson (1954) (15:3:12:10 by volume).

7.2.5 Peptide visualization

After air drying the maps, peptide spots were visualized by spraying with fluorescamine (Fluram, Hoffman-LaRoche) 0.001% w/v in 500 ml acetone and 1 ml pyridine and

viewed under U.V. light. This method is very sensitive and reacts with only 1-2% of peptide present. Alternatively 0.02% w/v ninhydrin in 500 ml acetone, 1 ml pyridine, 1 ml acetic acid was used. The map was allowed to develop overnight in the dark at room temperature. The map was then photographed or traced the next day. Spots for analysis were cut out, washed with acetone to remove excess ninhydrin and the peptide eluted for amino-acid analysis. The elution was carried out by finely slicing the paper, adding 6M HCl, mascerating and filtering under gentle suction with small additions of HCl.

7.2.6 Specific staining for arginine, histidine and tyrosine

If sufficient protein was available for several maps, specific amino-acid staining was carried out as follows:

Arginine Easley, (1965)

Equal volumes of the following reagents were mixed and sprayed on the chromatogram.

0.1% (w/v) 9:10 phenanthroquinone in EtOH.

10% (w/v) NaOH in 60% EtOH.

After reacting for 5 minutes at 50°C in an oven, arginine and its peptides were visualized as greenish spots under ultra-violet light. This stain could be followed by that for histidine.

Histidine (method of Pauly)

Solutions of 100 ml of 1% (w/v) sulphanilic acid in 1 M HCl and 1% (w/v) $NaNO_2$ in 100 ml of water were made up separately and chilled in an ice bath. A solution of 100 ml

of 10% (w/v) Na_2CO_3 was also chilled. The first two solutions were mixed and kept chilled. The alkaline solution was added and mixed with the $NaNO_2$/sulphanilic acid just before use and the chromatogram was sprayed in a well-ventilated hood. The colours appeared immediately but changed overnight to give a better differentiation between peptides containing histidine (deep red) and peptides containing tyrosine (brown).

7.3 Amino-acid compositions of Calcutta and normal LDH

The amino-acid analyses of LDH in the present work were performed on protein oxidised by performic acid. The analyses were calculated assuming a subunit M.W. of 35,000 per subunit for both LD_1 and LD_5 and 315 and 319 amino-acid residues respectively per subunit. These values were chosen to allow comparison with the analyses of other workers.

The half-cystine residues were determined in the form of cysteic acid and the methionine residues as the methionine sulphone. No correction was made for losses of threonine or serine, nor for tyrosine which might be under-estimated through oxidative losses. Neither was allowance made for leucine, isoleucine or valine which can be under-estimated due to slow hydrolysis where these residues are adjacent in the primary sequence.

Tryptophan was destroyed during acid hydrolysis and for the purposes of calculating the total number of residues of each amino-acid per protein subunit, a value of 5 for tryptophan was applied. Tryptophan shows little

variation among animal LDH's (Pesce et al., 1967; Holmes and Scopes, 1974) who found between 5 and 5.5 residues/subunit in 7 and 5 animal LDH's respectively in both LD_1 and LD_5.

The compositions of normal LD_1, Calcutta-homozygote LD_1 and normal LD_5 are shown in Table 7.1. Because of the limited amount of Calcutta LD_5 available, it was considered to be better utilized for peptide mapping and was not analysed. Total amino-acid analysis provides limited information on small differences in residue composition of similar proteins. The analytical precision of the amino-acid analyser method is accepted as ±3% when > 5 nanomoles/less abundant residue are present. This represents ± 1 residue at the level of 35 residues/polypeptide chain e.g. for an amino-acid such as Asx or Leu in LDH - Table 7.1. Unfortunately, of the charged residues of interest in this particular study, Asx, Glx and Lys analyse at about this level. A substitution in one of these quantitatively more common residues could easily pass undetected by total amino-acid analysis alone. Arginine, which analysed at about 10 residues/subunit - Table 7.1 is the remaining charged residue of interest. A single arginine residue change in the protein should be detectable since it represents approximately a 10% change in content, exceeding the analyser error of ± 3%.

Table 7.1 AMINO ACID COMPOSITIONS OF HUMAN LDH's

	LD_1			LD_5	
	Present work[a]	Pesce et al. (1967)	Calcutta-hom	Present work[b]	Wachsmuth et al.[c] (1964)
Lys	23.44 ±0.31	23.95 ±0.65	23.12	27.08	26.88
His	6.80 ±0.16	6.18 ±0.28	5.31	6.60	6.74
Arg	10.06 ±0.19	7.55 ±0.33	10.58	10.54	10.72
Asp + Asn	35.15	33.38 ±0.35	35.15	32.41	31.83
Thr	12.35 ±0.22	12.30*	12.73	13.25	13.35*
Ser	23.29 ±0.62	21.95*	24.35	21.89	21.54*
Glu + Gln	33.11 ±0.49	32.00 ±0.15	36.99	29.81	28.61
Pro	13.56 ±1.58	10.95 ±1.03	d	10.84	11.03
Gly	27.17 ±0.97	27.75 ±0.48	28.39	26.34	25.34
Ala	23.14 ±0.53	21.28 ±0.25	22.82	17.90	17.74
Cys[e]	5.14	N.D.	d	2.76	N.D.
Val	29.54 ±1.42	35.55*	22.95	29.10	33.06*
Met	5.88 ±1.16	8.13 ±0.05	d	8.58	7.72
Ile	17.19 ±0.55	21.88*	14.79	20.08	22.12*
Leu	33.39 ±0.53	33.75 ±0.60	31.55	39.03	36.46
Tyr	6.07 ±0.73	6.70 ±0.28	d	7.98	7.97
Phe	5.24 ±1.64	5.48 ±0.23	d	6.63	6.95

a. Mean and S.D. of 4 determinations; b. Mean of 2 determinations; c. Recalculated from Σ μ mole = 100%; d. Below the level of reliable integration, < 2 n.mole; e. Determined as cysteic acid; * Corrected values; N.D. not determined; LD_1 calculated to 314.5 residues/subunit, allowing 5 residues each for Try, Cys.; LD_5 calculated to 318.8 residues/subunit, allowing 5 residues each for Try, Cys.

7.4 Peptide mapping of Calcutta and normal LD_1

Several purifications of normal LD_1 were digested with trypsin and individually subjected to peptide mapping and these gave consistently comparable peptide maps. Staining for histidine, tyrosine and arginine was carried out on some of these maps.

Comparative maps of LD_1 normal and LD_1 Calcutta-homozygote are shown in Figure 7.1 and tracings of these maps showing the directions of electrophoresis and chromatography are shown in Figure 7.2. A number of spots which appeared to be involved in possible differences in the maps were selected and numbered as indicated on the tracing. The numbered spots from normal LD_1 which gave positive staining results are indicated in Table 7.2.

Table 7.2 LD_1 NORMAL. SPECIFIC AMINO-ACID STAINING

	Tr 1	Tr 2	Tr 5	Tr 6	Tr 8	Tr 9
Histidine		+			+	
Arginine	+	+	+	+	+	+
Tyrosine			+	+	+	

An extremely limited amount of Calcutta homozygote LD_1 was available for mapping. Of a total of 0.64 mg purified, about 0.14 mg was used for total analysis and 0.50 mg for mapping

Figure 7.1 Comparison peptide maps of normal and Calcutta LD_1. Electrophoresis pH 4.7, ascending chromatography - see Figure 7.2.

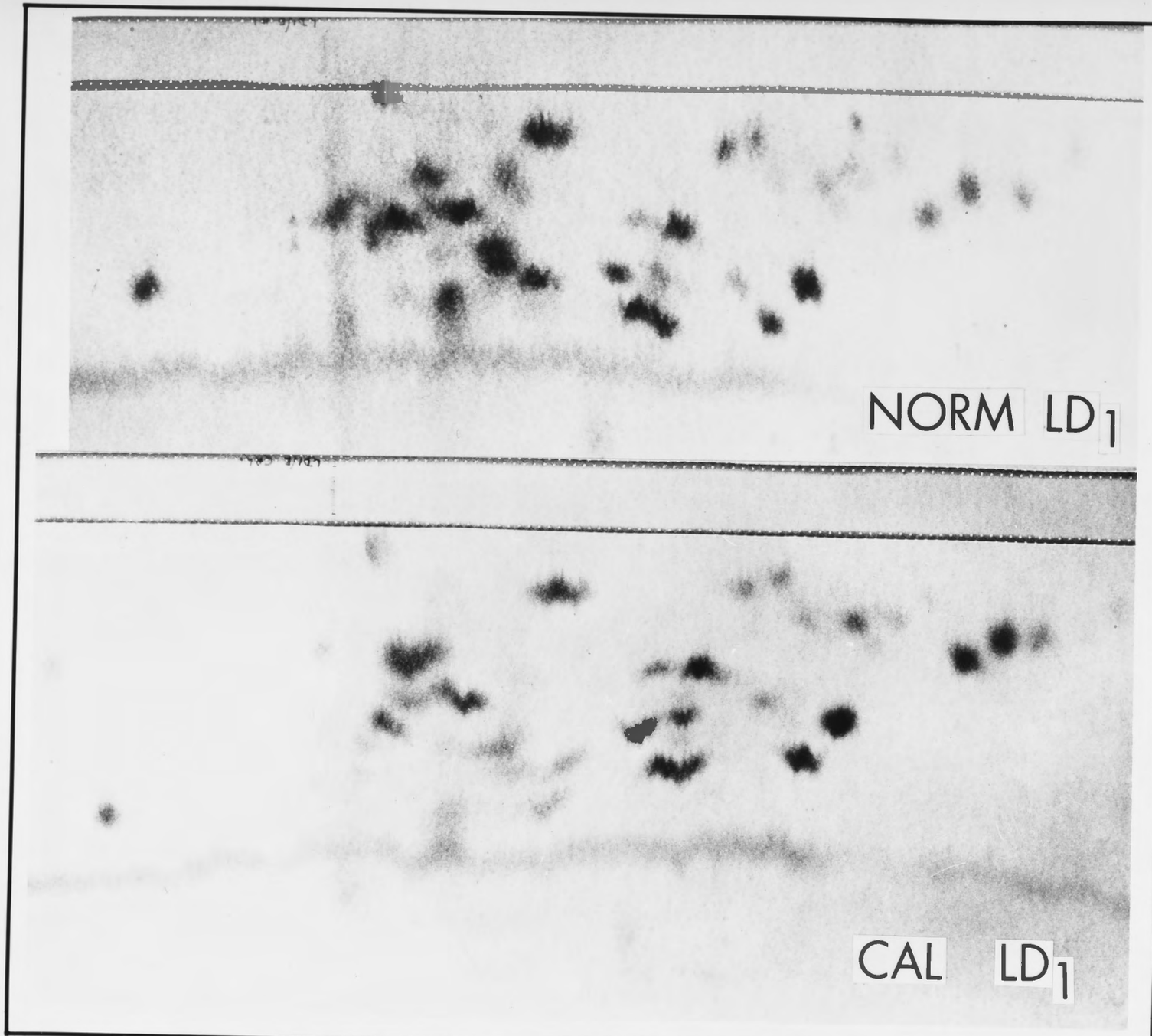
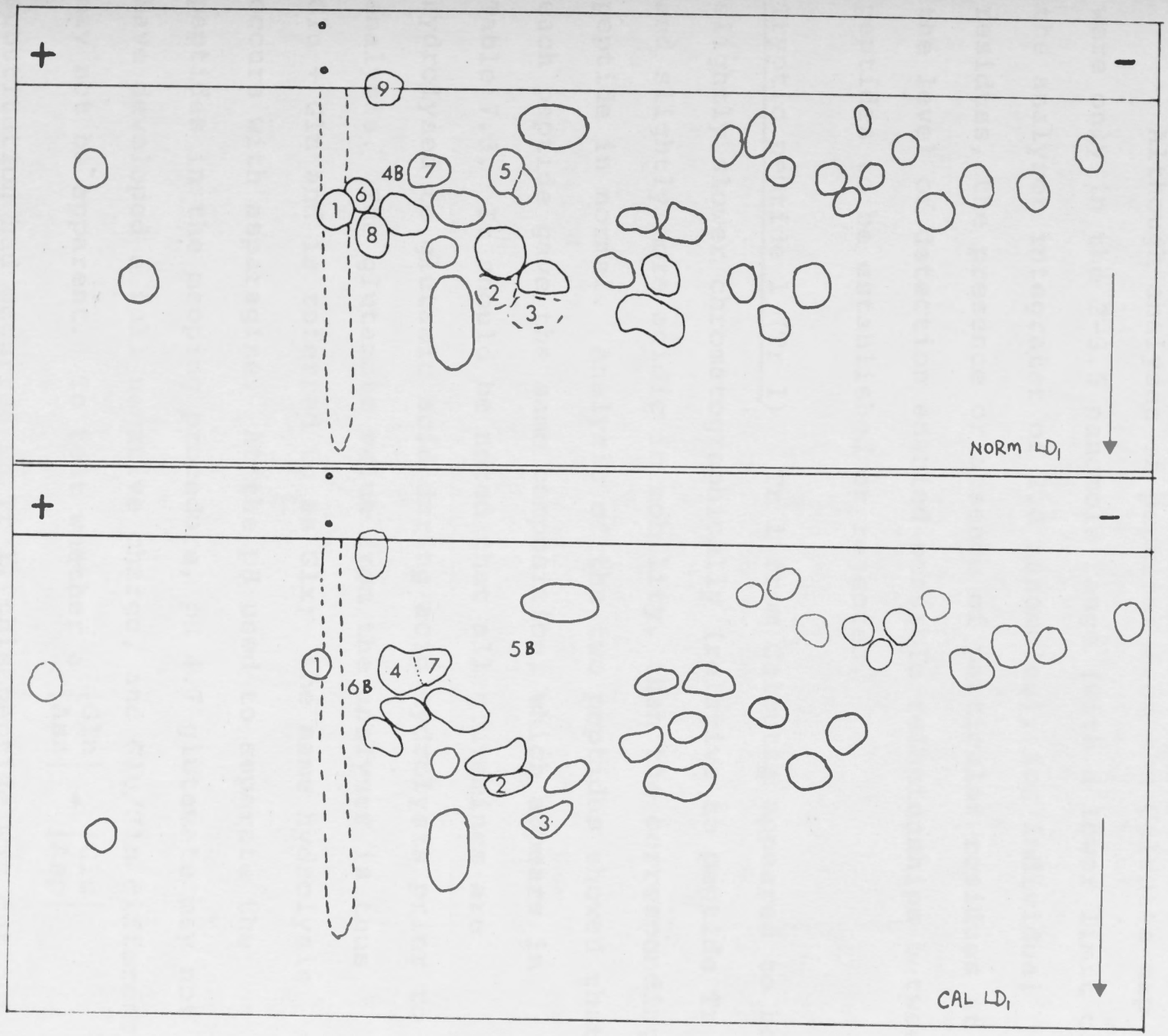

NORM LD1
CAL LD1

Figure 7.2 Peptide map tracings of normal LD_1 and Calcutta homozygote LD_1. Arrow indicates direction of ascending chromatography. Polarity of high voltage electrophoresis pH 4.7 indicated. This tracing refers to Figure 7.1.


+
-
9
4B
7
5
6
1
8
2
3
NORM LD1
+
-
1
6B
4
7
5B
2
3
CAL LD1

compared with nearly 10 mg normal LD_1 available for all analyses and maps.

7.4.1 LD_1 tryptic peptides analysed

Although analyses of peptides from the Calcutta map were only in the 2-3.5 nanomole range (with a lower limit of the analyser integrater of 2.0 nanomoles), for individual residues, the presence or absence of particular residues at the level of detection enabled possible relationships between peptides to be established or rejected.

Tryptic peptide 1 (Tr 1) Tr 1 from Calcutta appeared to be slightly slower chromatographically (relative to peptide Tr 7) and slightly more acidic in mobility, than the corresponding peptide in normal. Analysis of the two peptides showed that each peptide gave the same composition, which appears in Table 7.3. It should be noted that all glutamines are hydrolysed to glutamic acid during acid hydrolysis prior to analysis. The glutamate value from the analyser is thus Glu + Gln and is referred to as Glx; the same hydrolysis occurs with asparagine. At the pH used to separate the peptides in the mapping procedure, pH 4.7 glutamate may not have developed a full negative charge, and Glu/Gln differences may not be apparent. To test whether a $\left|\begin{matrix}\text{Gln}\\\text{Asn}\end{matrix}\right| \rightarrow \left|\begin{matrix}\text{Glu}\\\text{Asp}\end{matrix}\right|$ substitution had occurred in Tr 1, this peptide was cut out from a map of variant LD_1 purified from the heterozygote placenta. Tr 1 from this map, together with Tr 1 from a further normal LD_1 map were sewn to fresh paper and remapped

Table 7.3 ANALYSES OF TRYPTIC PEPTIDES OF LD_1 (expressed as molar ratios)

	Tr 1[a]		Tr 2	Tr 3	Tr 4	Tr 5		Tr 7		Tr 8	Tr 9
	N[b]	C	C	C	C	N	C	N	C	N	N
Lys							peptide absent				
His										+	
Arg	+					+				+	+
Asp + Asn	2.35	2.16			0.91	1.82		1.21	1.04	1.14	
Thr	0.17	-									
Ser	1.72	1.51	1.18		0.87	0.98					1.02
Glu + Gln	1.17	1.20	0.61	1.06	1.11			1.24	1.05	1.07	3.30
Pro											
Gly	1.87	1.52	1.04		0.75						1.05
Ala	0.98	1.00				0.98		0.65	<2n.mol	0.66	
Cys[c]	0.76	1.26									
Val	0.97	0.53						0.53	<2n.mol		
Met											
Ile	0.72	0.51		0.98							
Leu	0.94	0.97						0.86	<2n.mol	0.81	
Tyr						+				+	
Phe											
n.moles/ residue	6.0	3.8	3.0	2.0	3.0	2.0		4.0	2.0	3.0	4.0

a. For Tr 1 a technique involving NH_4OH chromatographic extraction of the peptide was used instead of the usual maceration technique since this peptide was of special interest; b. N = normal, C = Calcutta; c. Cys determined as cysteic acid; + refers to +ve staining result for histidine or arginine.

by the normal procedure, (except that the electrophoresis time was extended) to free Tr 1 from other "streak" peptides. From this new map each Tr 1 peptide spot was cut out, sewn to fresh paper adjacent to one another and electrophoresed at pH 8.9, 40 v/cm for 1½ hr and stained after drying with 1% ninhydrin. If Calcutta Tr 1 possessed an Asp or Glu residue substituted for Asn or Gln in the normal, then at alkaline pH, the acid would be completely ionized, carrying a full negative charge, while the amide would be uncharged. Calcutta Tr 1 should thus demonstrate a faster anodic migration than normal Tr 1. Both peptides migrated the same distance, eliminating a change of status of the two Asx residues or the possible Glx residue between the Calcutta peptide and normal peptide.

Tr 2 and Tr 3

These two peptides appear in positions chromatographically faster than two adjacent stronger (ninhydrin + ve) spots - see maps. Tr 3 appears nearly equal in intensity and clearly separated from its adjacent spot in Calcutta but a Tr 3 spot of equivalent intensity is absent in normal. A similar argument applies to Tr 2. Tr 2 and Tr 3 from Calcutta were analysed (Table 7.3). Tr 5 from normal is absent in Calcutta and the possibility of Tr 5 giving rise to Tr 2 and Tr 3 in Calcutta was considered to explain this absence. Clearly the sum of the analyses for these two peptides does not account for the Tr 5 analysis which contains a tyrosine residue.

Tr 4

Tr 4 appears as an extra spot on the Calcutta map, merging with Tr 7 and is absent from the normal map (4B = 4 blank). Tr 7 from normal contained Ala, Val and Leu not found in Tr 4 of Calcutta, which contained serine not detected in Tr 7 from normal. These differences in analyses justify the arbitrary division of the 4-7 spot in Calcutta into Tr 4 and Tr 7.

Tr 5

Tr 5, eluted from the normal map, is a tyrosine containing peptide which is absent on the Calcutta map (5B). The possibility that Tr 5 from normal had by an acidic mutation migrated to the position occupied by Tr 4 from Calcutta was considered. This possibility was rejected on examination of the respective analyses. Tyrosine and alanine found in Tr 5 are absent in Tr 4, which contains Glx and Gly not found in Tr 5. These two peptides are not related by a simple acidic mutation.

7.4.2 Nanomole level micro-method on Polygram 300 sheets

The purified LD_1's from normal and variant were dialysed and lyophilised as before. Performic oxidation of the proteins was not used for this method. Tryptic digestion was carried out in the normal way except that three additional lyophilizations in 10% isopropanol were performed as detailed in Vanderkerkhove & Vanmontagu, 1974. Freshly distilled water, distilled on the day of use was always used.

Fingerprints were prepared by spotting ∿ 35 ug samples of the digested protein onto Machery:Nagel Polygram MN 300 sheets 0.1 × 200 × 200 mm, using microcapillaries, drying with an electric hair drier. The sheets were electrophoresed at 900 V, 25 mA for 40 minutes in pH 3.5 pyridine/acetic acid/water (1:10:89) then air dried in an oven, briefly at 50°C. Ascending chromatography in butanol/acetic acid/water/pyridine of the previously used composition for ∿ 3½ hours at room temperature followed. Peptides were visualised by spraying with Fluram 0.01% w/v in acetone as previously described. The maps were immediately visualised under ultraviolet light and photographed on Ilford HP5 400 ASA film.

Two purifications of normal LD_1 and two purifications of Calcutta-1 LD_1 were treated as described and mapped. The two maps of normal LD_1 from separate purifications were consistent with each other. Calcutta heterozygote LD_1 was mapped three times from the two purifications and yielded comparable maps with a similar distribution of spots to the normal LD_1 maps, Figure 7.3. These nanomole level maps provided superior resolution of the peptides in the neutral region compared with the paper peptide maps.

A single extra spot was observed on the Calcutta-1 maps. This additional tryptic peptide is fast chromatographically and has electrophoretic mobility ½B where B is the mobility of the most basic peptides. It is of much lower intensity than the most intense spots. Although it is appreciated that there are many reasons why one spot may have less intensity than another on Fluram staining such as the

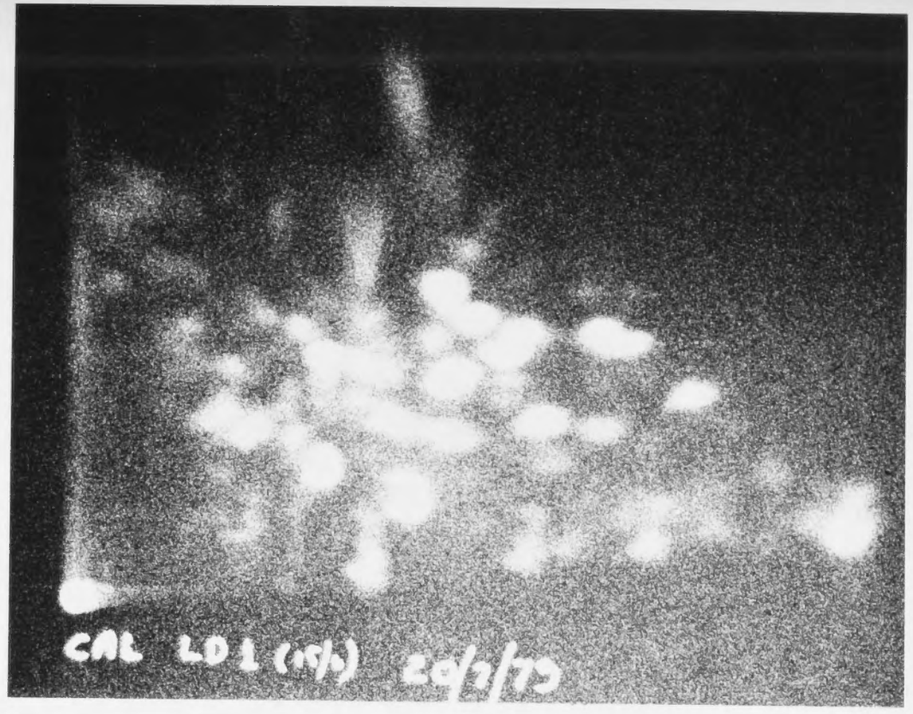
Figure 7.3 Comparison tryptic fingerprints of normal and Calcutta-het. LD_1.

A and B are different exposures of the same normal LD_1 fingerprint at the left hand side. Equivalent exposures of the same Calcutta-het. LD_1 map are to the right hand side for comparison.

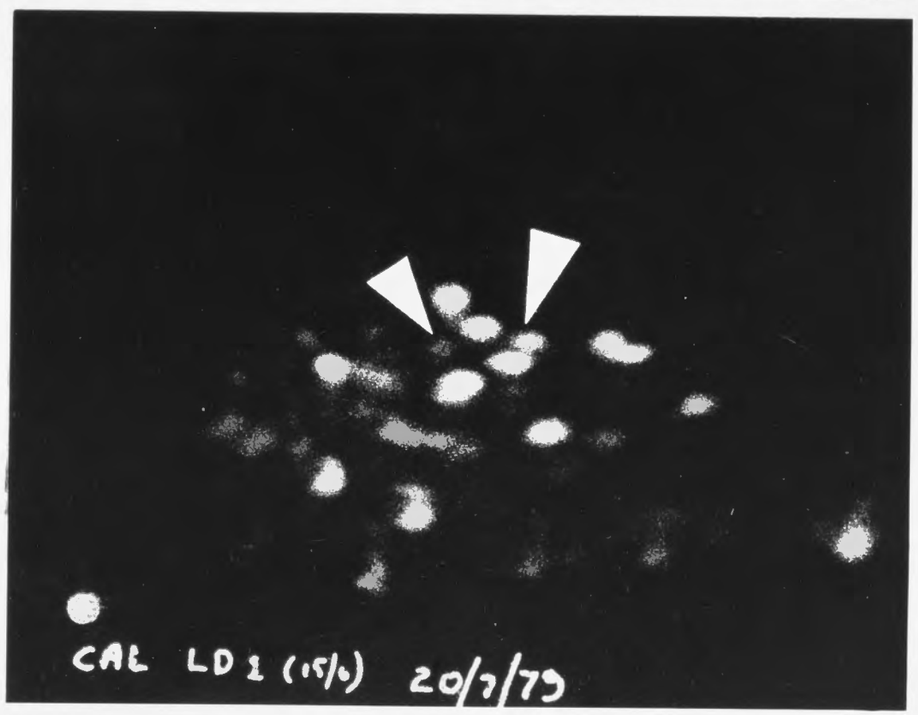
In B exposure, Cal fingerprint, the left hand arrow marks the extra Calcutta peptide. The right hand arrow marks the "sister" peptide.

Mapping details: Origin spot at bottom LHS of each map. Electrophoresis pH 3.5 toward cathode at RHS. Ascending chromatography towards top of each map. Polygram MN 300 sheets, Fluram staining, photographed under ultra-violet light.


NORM LD1 22/7/79 (R)
A


CAL LD1 (1%) 20/7/79


NORM LD1 22/7/79 (R)
B


CAL LD1 (1%) 20/7/79

number of nitrogen containing side chains, the extra peptide might be derived from only half the polypeptide chains. Since heterozygote LD_1 was used the extra peptide might originate from only the β chains while all other spots derive from both B and β chains. A "sister" peptide with reduced intensity but normal position was also observed on the Calcutta map. In Figure 7.3 a normal LD_1 fingerprint on the left hand side is compared with a Calcutta-heterozygote LD_1 fingerprint on the right, at two exposures, A and B. The extra Calcutta peptide is indicated by the left hand arrow on the Calcutta B map. Examination of the equivalent region in the normal LD_1 map reveals no peptide spot. The prints of the same maps in A were deliberately over-exposed to highlight this region of the map and enable close comparison. The extra peptide spot was clearly seen on the Calcutta A map and absent on the normal A map.

The other arrowed spot, on the right on the Calcutta B map is the putative sister peptide of the extra Calcutta spot. It will be noted that the chromatographic mobility of the two arrowed spots is nearly identical, which may indicate a similar composition. They differ in that the extra spot is more acidic in its electrophoretic mobility.

An intensity comparison of the "sister" spot as it appears on the Calcutta map was made with the equivalent spot on the normal map. When this spot is compared in intensity with its two nearest neighbours (with which it forms a triangle of three spots) it can be observed to be reduced in its relative intensity to these spots on the Calcutta B map.

When the same intensity comparison is made on the normal B map, each of the three peptide spots is of about the same intensity. This observation suggests that peptide from the "sister" peptide is being lost to the extra Calcutta peptide. These two arrowed peptides should be related by the same amino acid sequence except for the substitution mutation. To summarise the argument, the extra spot is derived only from β chains while the "sister" spot is derived only from B chains.

An attempt was made to excise and analyse these peptides, pooling the material from the three Calcutta maps. Recovery was not good and the suspected relationship to the normal peptide could not be established.

A substituted peptide which could account for the fast anodal migration of the Calcutta-1 variant should contain one of the following types of mutation ...

a. Neutral ⟶ acidic (gain of one negative charge)

b. Basic ⟶ acidic (effectively gain of two negative charges)

c. Basic ⟶ neutral (loss of a positive charge)

The extra peptide observed eliminates option c, since loss of an arginine or lysine basic residue would mean one fewer tryptic site and therefore one fewer peptide spot on the Calcutta map. The same argument applies to option b. As well it has been argued that insufficient change in pI has been observed in the isoelectric focusing result to account for a two charge change as in option b.

An amino acid substitution of the type a would explain the observed result and could occur by a single base change in the affected codon.

Asparagine ⟶ aspartate

or Glutamine ⟶ glutamate.

7.4.3 Conclusions

Some apparent differences between the Calcutta and normal LD_1 paper maps were investigated and possible explanations examined. The "disappearance" of certain spots may be explained by the overlaying of spots, caused by slight variation in mobility of peptides especially in the region of limited electrophoretic and chromatographic mobility near the origin.

the origin. A full analysis of all spots in the neutral region would be necessary to check this possibility. The region of basic peptides to the right hand side of the maps appears highly comparable. However differences occur in the crowded, less well-resolved region of neutral peptides in the region just cathodic to the origin. The possibility remains that a peptide carrying the Calcutta mutation is located in this region.

7.5 Peptide mapping of Calcutta and normal LD_5

Comparison maps of LD_5 purified from normal and Calcutta placentae are shown in Figure 7.3. Tracings of these maps showing the numbered peptides appear in Figure 7.4. The protein loadings on the compared maps are each approximately 1 mg. A higher loading (3 mg) normal LD_5 map is shown in Figure 7.5 and proved useful for checking analyses of normal peptides.

7.5.1 LD_5 tryptic peptides analysed - Tr 1 and Tr 2

Fluram staining showed a strongly fluorescing region in the central "streak" region which proved to be only weakly ninhydrin positive. The large lobed region was divided into two spots Tr 1 and Tr 2. On the Calcutta map Tr 2 appeared to fluoresce more strongly than Tr 1 and to separate from Tr 1 more distinctly compared to the equivalent normal structure. When removed from the respective maps and analysed (Table 7.4) Tr 1 and Tr 2 were clearly distinct arginine tryptic peptides, the most notable difference was that Tr 1 contained a cysteic

Table 7.4 ANALYSES OF TRYPTIC PEPTIDES OF LD_5 (expressed as molar ratios)

	Tr 1		Tr 2		Tr 3		Tr 4	Tr 5	Tr 6	Tr 7
	N[a]	C	N	C	N	C	N only			N only
Lys										
His					1.06	1.05	0.79			
Arg	0.84	1.00	0.90	0.92						
Asp + Asn	2.19	1.71	0.48	<2n.mol	1.73	1.76	1.06			0.82
Thr	-	0.39[c]	1.61	1.89	0.46	0.51				
Ser	1.93	1.77	1.25	1.25	1.09	1.03	1.15			
Glu + Gln	-	0.64[c]	2.55	3.11						
Pro										1.05
Gly	2.09	1.78								
Ala	1.05	1.03	1.01	1.02	0.44	0.48				1.18
Cys[b]	1.01	0.85			0.66	0.57				
Val	<2n.mol	0.44	0.78	0.84	0.52	0.40				
Met										
Ile	<2n.mol	0.35								0.93
Leu	1.10	1.06	1.07	1.06						
Tyr										
Phe					0.85	0.91				
n.moles/ residue	3.27	6.65	4.51	3.48	4.44	6.90	3.00	< 2n.mol	< 2n.mol	5.43

a. N = normal, C = Calcutta
b. Cysteine determined as cysteic acid
c. Possibly background contamination.

Figure 7.4 Comparison peptide maps of normal and Calcutta LD_5. Electrophoresis pH 4.7, ascending chromatography - see Figure 7.4.

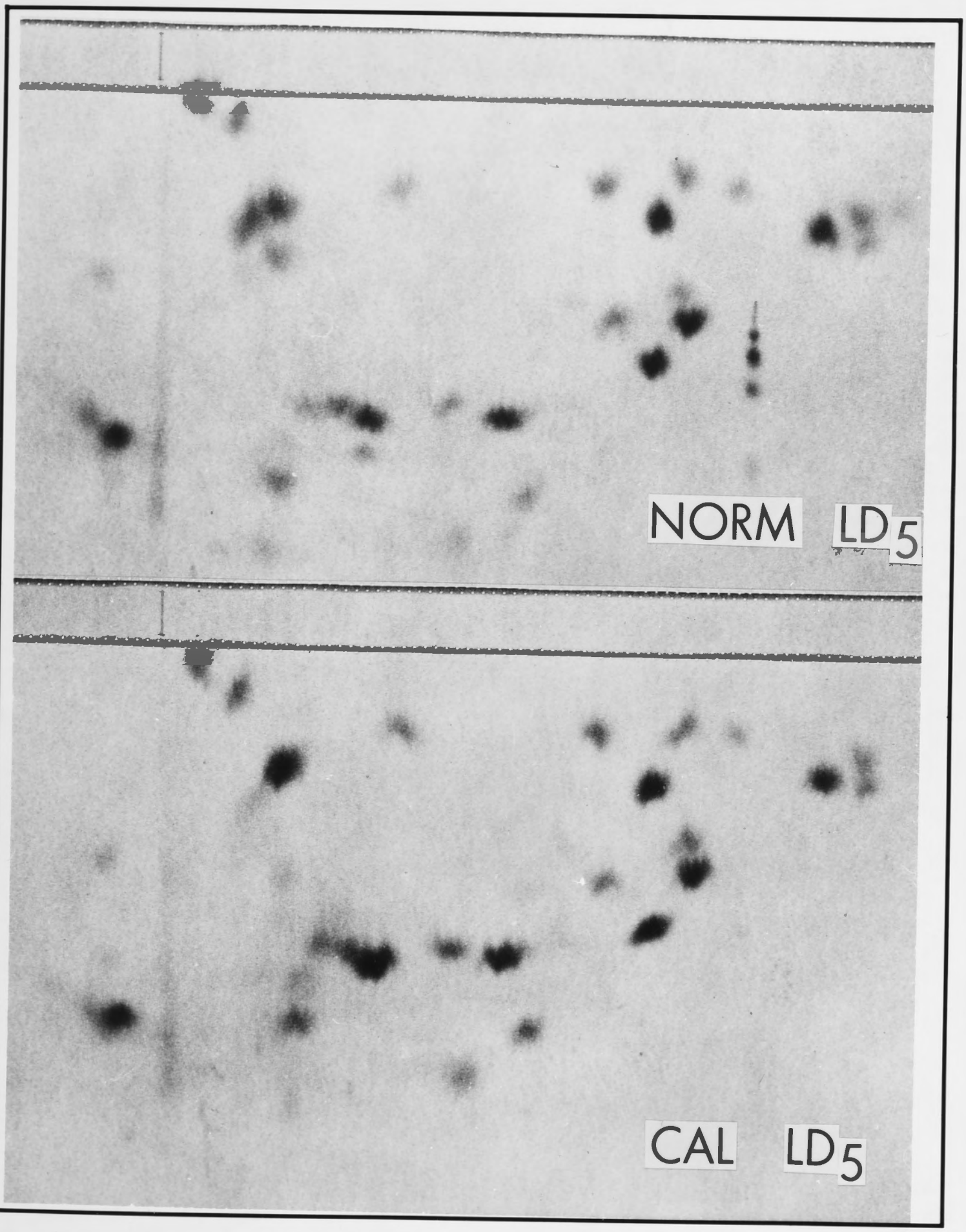

NORM LD5
CAL LD5

Figure 7. 5 Peptide map tracings of normal LD_5 and Calcutta heterozygote LD_5. Arrow indicates direction of ascending chromatography. Polarity of high voltage electrophoresis pH 4.7 indicated. This tracing refers to Figure 7.3.

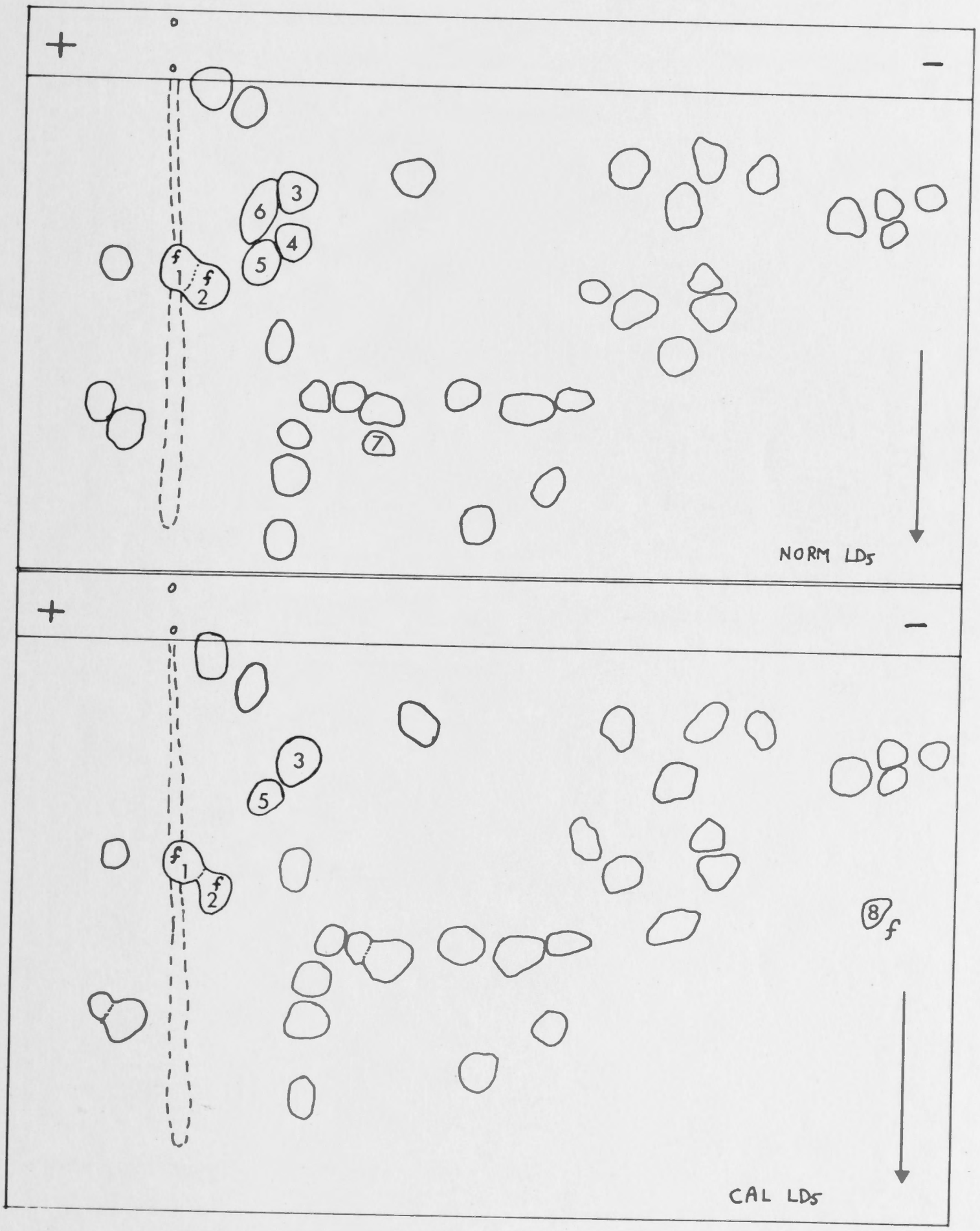
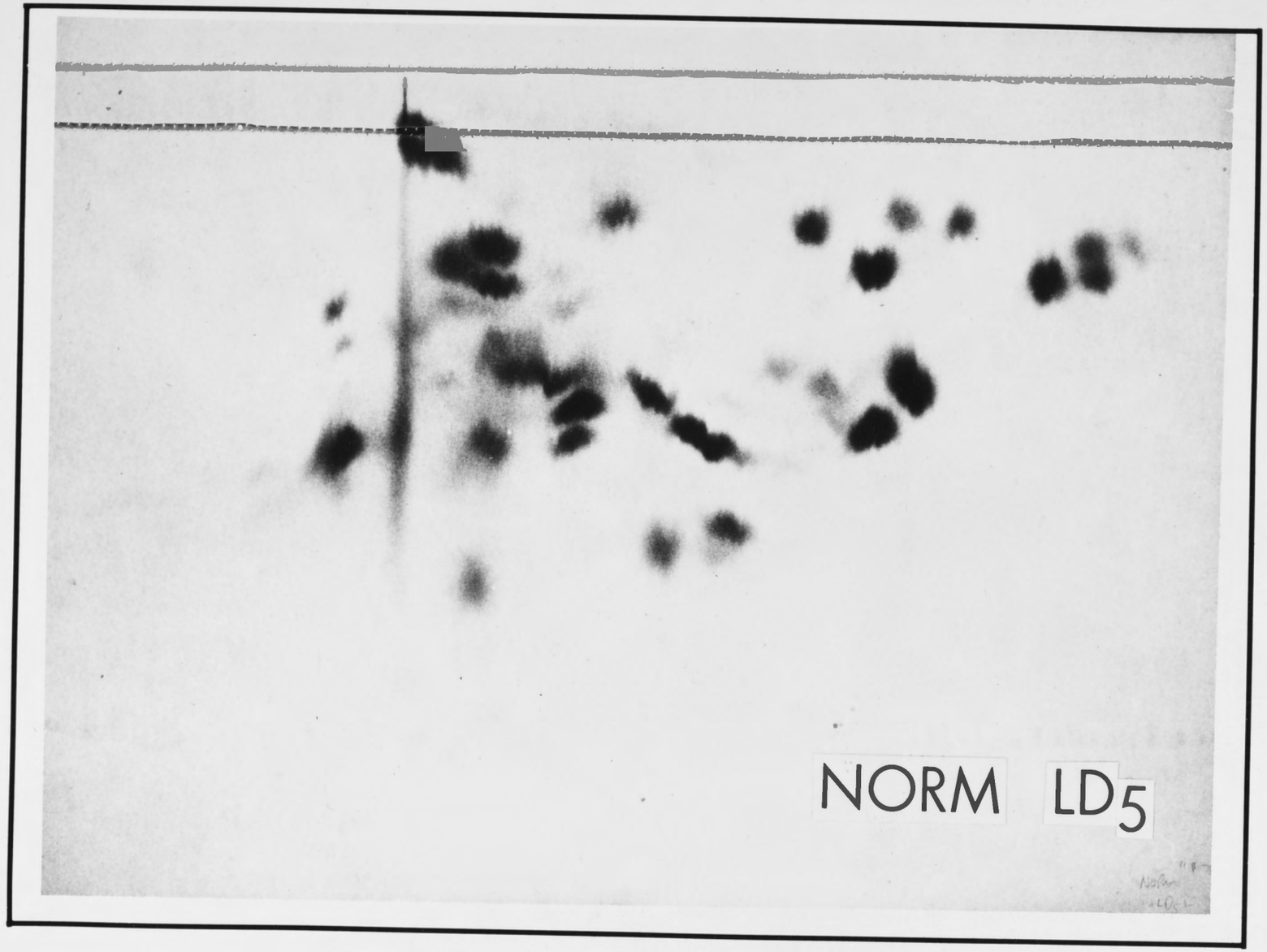

+
−
3
6
4
5
f
1
f
2
7
NORM LD5
+
−
3
5
f
1
f
2
8
f
CAL LD5

Figure 7. 6 Peptide map of normal LD_5. 3 mg loading. Electrophoresis polarity and chromatography as for Figure 7.4 normal LD_5.


NORM LD5

acid residue. No significant differences between the respective Tr 1 peptides of Calcutta and normal, or the respective Tr 2 peptides were evident. LD_5 Tr 1 shares a common position on the map with Tr 1 from LD_1 mapped under the same set of conditions. The analyses of Tr 1 from LD_1 and Tr 1 from LD_5 correspond, suggesting that this common peptide may be the common active site peptide containing cysteine 165 and arginine 171 (Dayhoff, 1972). The sequence of the active site peptide starts Val-Ile-, a dipeptide which is known to hydrolyse slowly. Incomplete recovery of Val and Ile from the 22 hr acid hydrolysis used could account for the low yield of these two residues in the analyses of LD_5 Tr 1 - Table 7.4.

Tr 3

Tr 3 was chosen as a reference peptide initially to compare loadings and recovery levels of peptide from each map. The sum of 28.16 nanomoles of this peptide was recovered from the normal peptide spot and 42.88 nanomoles from the Calcutta peptide. Tr 3 shared a common analysis for normal and variant each possessing a tyrosine and a cysteine residue.

Tr 4

Tr 4 appeared as a ninhydrin positive peptide on the normal map which was absent from the Calcutta map. Analysis showed it to be a small lysine peptide. The possibility that Cal "3" peptide was in fact a mixture of normal Tr 3 plus Tr 4

overlaying it was considered especially since Calcutta "3" appeared to stain relatively more strongly than normal 3. This possibility was excluded however even though Tr 3 and Tr 4 share a number of common amino-acids since the molar ratios of the residues Cys and Tyr do not fall significantly in the Calcutta "3" analysis relative to the residues which are common to both peptides. Tr 4 represents a real tryptic peptide absent in Calcutta LD_5.

Tr 5 and Tr 6

Strongly fluorescing spots in the position numbered 5 were seen following Fluram staining on each map. This weakly ninhydrin positive region gave an analysis indistinguishable from background at < 2 nanomoles compared with Tr 3 ∿4-6 n.moles. Region 6 on the normal map, although ninhydrin positive, when analysed did not possess a lysine or arginine at the levels recovered from the other tryptic peptides analysed. It was concluded that 5 and 6 did not represent tryptic peptides and spot 6 is not a real peptide difference between Calcutta and normal.

Tr 7

Tr 7 peptide was present on the normal map but not on Calcutta. Analysis (Table 7.4) showed it to be a small arginine peptide containing methionine. Recovery at the 2.8 nanomole/residue level from the normal LD_5 map was checked by analysing the same spot from the higher loading normal map, at the 5.43 nanomoles/residue level, which corroborated the original analysis.

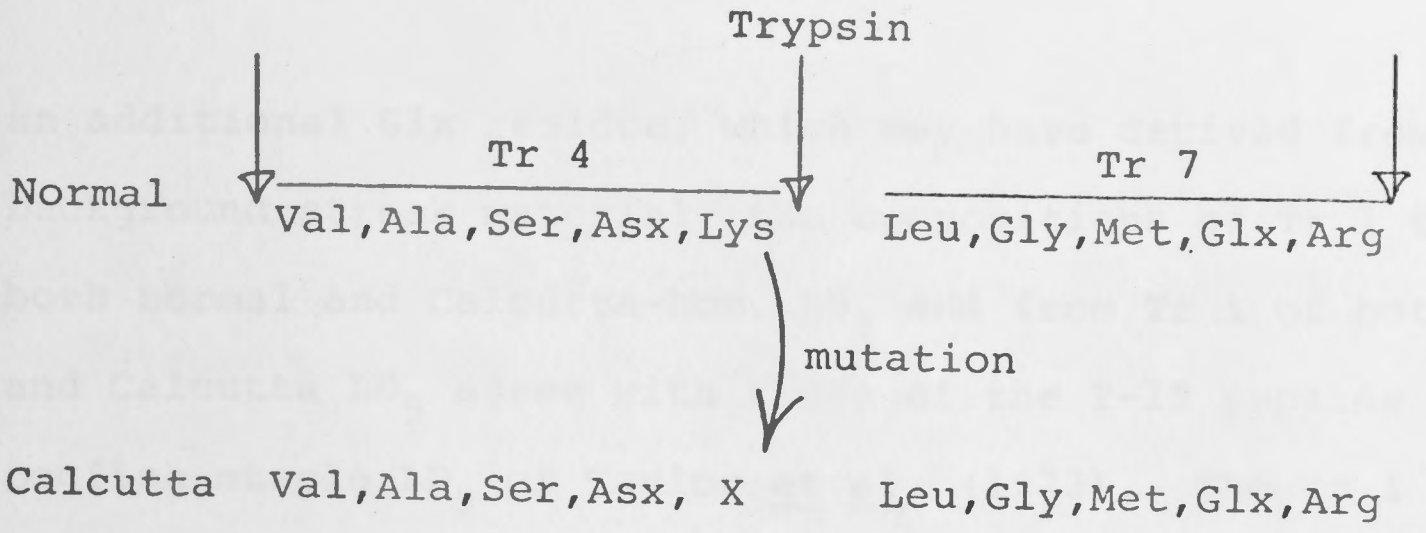
Tr 8

Spot 8 was a compact brightly fluorescing ninhydrin negative region of strongly basic mobility. The analysis did not confirm it as a tryptic peptide, although ammonia was detected at nearly 3 times the level normally associated with the other analysed peptides. This analysis and mobility suggests a low molecular weight compound, possibly from ammonium acetate.

7.5.2 Conclusions

Analysis of a number of selected ninhydrin and Fluram positive spots eliminated several apparent differences between the peptide maps of Calcutta and normal LD_5. Two tryptic peptides Tr 4 and Tr 7 were detected on the normal map (and confirmed on the higher loading map) which were absent from the Calcutta map. Since the Calcutta LD_5 protein was purified from a heterozygous source, some Tr 4 and Tr 7 from normal polypeptide chain A might be expected. This was not observed even though the very sensitive Fluram staining was used and suggests that only Calcutta polypeptide was present. The presence of a single Calcutta LD_5 band on zone electrophoresis under a variety of conditions - Chapter 3, may mean that at least some A-α associations do not occur in Calcutta LD_5.

The presence of two peptides in normal, absent in Calcutta suggests the loss of a tryptic site from a larger "4 + 7" peptide e.g.



Such a larger peptide with one less basic residue would be located to the acid side of spot 4 and might well be located in the neutral "streak" region. Fluram and residue specific stains indicate that several peptides are contained in the "streak". A basic to neutral (or acidic) substitution is consistent with the extra negative charge suggested by the zone electrophoresis of Calcutta native protein - Chapter 3.

The other possibility is that a sequence or sequences involving Tr 4 and Tr 7 have been deleted from the LD_5 polypeptide. Failure to locate the larger "4 + 7" peptide would enhance this postulation. Provided one or both of Asx and Glx were the amide residues, a deletion of these peptides would mean the loss of an arginine plus a lysine from Calcutta accounting for its anodic electrophoretic separation in the whole protein. The SDS/acrylamide gel study of LD_5 molecular weights - Chapter 3 - is probably unable to detect a deletion of 10 residues in 330 (3%).

7.6 The active site peptide

The amino-acid sequence adjacent to Cys 165 has been strongly conserved in all vertebrate LDH's examined and is homologous with the equivalent region in other enzymes in the dehydrogenase family - Chapter 1. With the exception of

an additional Glx residue, which may have derived from the background streak material, the compositions of Tr 1 from both normal and Calcutta-hom. LD_1 and from Tr 1 of both normal and Calcutta LD_5 agree with those of the T-19 peptide from Dogfish muscle LD_5 of Taylor et al. (1973). The Tr 1 compositions from each of the human enzymes analysed is shown aligned with the known sequences of the active site peptide (Dayhoff, 1972).

	165 171
Pig LD_1	Val-Ile-Gly-Ser-Gly-Cys-Asn-Leu-Asp-Ser-Ala-Arg
Beef LD_1	Val-Ile-Gly-Ser-Gly-Cys-Asn-Leu-Asp-Ser-Ala-Arg
Normal LD_1 human	Val,Ile,Gly,Ser,Gly,Cys,Asx,Leu,Asx,Ser,Ala,Arg(Glx)
Calcutta LD_1	Val,Ile,Gly,Ser,Gly,Cys,Asx,Leu,Asx,Ser,Ala,Arg(Glx)
Normal LD_5	Val,Ile,Gly,Ser,Gly,Cys,Asx,Leu,Asx,Ser,Ala,Arg
Calcutta LD_5	Val,Ile,Gly,Ser,Gly,Cys,Asx,Leu,Asx,Ser,Ala,Arg

Both Cys-165 and Arg-171 are essential residues at the enzyme active site, arginine interacting directly with the pyruvate carboxyl. This active site peptide is one of only 5 cysteine containing peptides in LDH. According to the sequence of Dogfish muscle LD_5 (Taylor et al., 1973) the other 4 cysteines are contained in peptides of 20,23,31 and 4 residues length. These other Cys containing peptides are identifiable by their size and by their less common marker residues, histidine, methionine, proline and aromatic residues. Of the four cysteine peptides, the three larger ones are all lysine peptides, and only T 29, the tetrapeptide terminates in arginine.

It appears evident from these considerations that Tr 1 from human LDH in this work is probably the highly conserved active site peptide and is therefore a particularly unlikely candidate to account for the Calcutta mutation.

7.7 General conclusions

The limitations of attempting to identify amino acid changes in protein variants with minimal quantities of pure protein are clearly evident from this work. Differences from the normal proteins were observed in both variant LD_1 and LD_5 maps by the paper method but rigorous authentication of these apparent differences requires further supplies of pure protein and further mapping. When an additional 300 ml of whole blood (about 50 ml of packed red cells) became available, a nanomole method on Polygram MN 300 plates was used. This method proved superior in the resolution of LD_1 tryptic peptides and a pair of peptide spots apparently related by an anodic substitution were observed.

Had tryptic mapping failed to indicate a difference peptide, it was intended to follow tryptic digestion with thermolytic digestion prior to the nanomole method of mapping. Thermolytic digestion would further cleave the tryptic peptides at hydrophobic residues to yield, on average, tripeptides, instead of the decapeptides, on average, of a tryptic digest. The resultant fingerprint would have been far more crowded (120 spots approximately) instead of the 35 obtained after tryptic cleavage and fingerprinting. This additional thermolytic digestion was not found to be necessary or possible with the amount of protein available.

8. DISCUSSION

With one exception, genetic variants of LDH, in man, are relatively rare. The exception is the variant Calcutta-1 which forms the subject of the present investigation.

In the Introduction (Section 1.4.1) the comprehensive review of population studies indicates that heterozygote individuals carrying the Calcutta-1 mutation are widely distributed in India, but have been detected in only a very few cases outside the Indian sub-continent. In addition three persons homozygous for the Calcutta-1 mutant have been found, two of them siblings living near Bombay and the third is an unrelated individual living in South India. All these homozygous persons were healthy adults.

The widespread distribution of a mutant allele, such as Calcutta-1, in a geographically circumscribed population raises important fundamental genetic questions: where did the mutation arise? does it have any selective advantage enabling it to spread in the population? are there any demonstrable differences in physiological and/or biochemical parameters associated with the mutant which might explain its advantage? and what are the structural differences between the normal and the mutant gene product?

The present study has focused attention on the latter two questions. Although further work is needed to make possible complete answers to these questions, it is clear that there are significant differences in some biochemical parameters

and in structure. Finally the question of whether the Calcutta-1 mutation is in fact an LDH A or B subunit mutation has been examined in some detail and the conclusion reached is that the evidence presented suggests that Calcutta-1 is a B subunit mutation. However, epigenetic changes which may affect isoenzyme phenotype have been considered in relationship to the Calcutta-1 phenotype.

8.1 Epigenetic events affecting protein phenotype

Several kinds of event affecting protein heterogeneity are recognised which are considered to be non-genetic, that is, not due to changes in the protein primary structure encoded in the gene. This heterogeneity may result from specific modifications of the polypeptide chains or certain of its side chains following the translation step, or may be brought about subsequently during the activation, maturation or ageing processes of the protein at its functional site. Modifications which chemically alter the polypeptide following translation are known as epigenetic changes or post-translational changes. Frequently the charge on the protein is altered affecting its electrophoretic mobility. Modified protein components may be represented as sub-bands adjacent to the major protein zones on gels or as additional eluted peaks on ion-exchange columns. The major types of epigenetic modification will be considered.

Amino terminus changes

The functional group at the N-terminus of proteins at physiological pH is a positively charged α-amino group

($-NH_3^+$). A number of different changes, neutralizing this charged group have been reported which result in an increased anodal mobility on electrophoresis.

Acetylation of the free amino terminus has been recognized in Soyabean Leghaemoglobin (Lb), (Whittaker *et al.*, 1979). Two components, Lb_a and Lb_b exhibit distinct isoelectric points, yet are homologous except for the N terminal tryptic peptide. The authors suggest that Lb_a ... NH_3^+-Val-Ala- gives rise to Lb_b ... NAcetyl Ala- *in vivo* by a post-translational cleavage of the N-terminal valine followed by acetylation of the new terminal alanyl residue. In human foetal haemoglobin (HbF_1), N-acetylation of one of the two γ chains has occurred (Schroeder & Jones, 1965). N-acetylation is not uncommon amongst intracellular enzymes. All known vertebrate LDH N-terminal sequences, both LD_1 and LD_5, shown in Dayhoff, (1972) are N-acetyl blocked. HbA_{1c} is a normal minor component representing 5-7% of total adult human Hb. Holmquist & Schroeder (1966) showed that the N terminus of one of the two β chains in HbA_{1c} was modified by a Schiff base condensation with a long chain aldehyde or ketone. Other types of N terminal modification are found in proteins which possess N-formyl methionine which is later removed at protein activation. N-terminal bound pyridoxal phosphate glutamate-aspartate transaminase has been reported by Hughes *et al.*, (1962).

As well as epigenetic changes involving the α-amino terminus, lysine ε-amino groups and histidine imidazoles may be modified. Minor components of *Mus musculus* Hbb-d and Hbb-p bands were observed *in vitro* (Morton & Tobin, 1977), after the protein was exposed to maleate buffer. These

authors showed that a single lysine residue at β76 was alkylated in maleate buffer to yield N- (2succinyl) lysine, resulting in faster anodal mobility on electrophoresis. Trayer et al.,(1968) showed that N-methylation was a normal maturation process in the transition from foetal to adult forms of contractile proteins. N-methylation was activated in skeletal muscle affecting both myosin and actin resulting in specific residues being converted to 3-methyl histidine. In pure actin approximately one residue in nine was methylated, conferring non-polar character on the imidazole ring. The flagellar protein, flagellin from Salmonella typhimurium was found to possess N-methyl lysine at approximately 50% of the lysine residues along the chain (Ambler & Rees, 1959). In the structural protein, collagen, hydroxylated lysine is a normal and quantitatively important residue. The foregoing examples indicate the variety of changes affecting the basic residues and the N-terminus of proteins which eliminate their positive charge and will lead to enhanced anodal mobility on electrophoresis of the affected proteins.

Deamidation of Glutamine and Asparagine side-chains

Cytochrome C is found in several forms in some mammalian tissues. Margoliash & Fitch, (1968) found each of these forms to have the same primary sequence, suggesting only a single locus for cytochrome C in a particular organism. Modified forms of this protein were thought to arise by deamidation of some of the glutamines and some of the asparagine residues.

Sulphydryl modification

It has been reported by Fritz & Jacobson (1965) that electrophoresis of LDH isoenzymes of some animals, in the presence of 2-mercaptoethanol (2-ME), can result in variable banding patterns. From 5 to 8 "sub-bands" could be seen after electrophoresis of mouse muscle LDH, but rabbit muscle LD_5 was unaltered. A result suggesting various degrees of NAD binding from 0 to 4 molecules of NAD per tetramer of LDH when NAD was added, was not found to be reproducible. Rabbit LDH mobility was increased in the presence of 10 mM NAD but the banding pattern was not altered. While -SH group involvement was thought by these authors to be involved, no single explanation, variable NAD binding or -SH group modification, was adequate to account for the observations. It was considered that the explanation of the "sub-banding" phenomenon might be species specific.

Alterations in mobility of the MDH isoenzymes of the marine snail Ilyanassa obsoleta after electrophoresis on starch gels preceded by 2-ME exposure, were observed by Meizel & Markert (1967). Two distinct MDH's were found - mitochondrial MDH (MDH_m) and supernatant MDH (MDH_s), possessing different primary structures and kinetic properties. MDH_m was unaffected by 2-ME but MDH_s could be converted from an anodal band to a cathodal band by prior 2-ME treatment. As many as seven isoenzyme bands could be reversibly changed to a common form. The original pattern was regained after the removal of 2-ME and oxidation of the

-SH groups by exposure to air. It was thought that various intramolecular disulphide bridges were reduced by the 2-ME resulting in the slow interconversion of the protein to one major form. A similar effect was seen with pig heart MDH when a 3-banded pattern could be changed to a 2-banded pattern by 2-ME treatment.

Certain erythrocyte enzymes were found to be susceptible to changed activity and electrophoretic mobility by treatment with oxidized glutathione (GSSG), (Walter & Caccam, 1966). Aspartate aminotransferase was caused to migrate more rapidly toward the anode on electrophoresis after reacting with GSSG but it was suggested that this altered mobility was due to an absorption phenomenon not to mixed disulphide formation. The isoenzymes of human erythrocyte LDH and of horse erythrocyte G6PD showed unchanged mobilities and specific activities after GSSG treatment. Reduced glutathione (GSH) is present in many cells including human erythrocytes. The anodal component of human haemoglobin, HbA_3 results from Hb interaction with GSH (Schroder & Jones, 1965). Many other examples of heterogeneity in proteins involving or implicating -SH groups are to be found in the literature. Sulphydryls are readily oxidised *in vivo* and *in vitro* particularly in the sulphur-rich proteins such as transferrin and in enzymes dependent on a cysteine at the active site. Precautions against *in vitro* oxidation need to be observed in handling such proteins to avoid generating "extra" anodal bands. These usually involve the controlled addition of 2ME or

dithiothreitol (Clelands reagent) to keep protein -SH groups reduced.

Proteins such as the immunoglobulins whose tertiary structure is dependent on intra-chain disulphide bridges undergo this oxidation process following translation. As the protein is synthesized, juxtapositioning of the cysteine residues must allow favourable contact for the covalent bond of cystine to form between sulphur atoms. In addition to oxidation, certain exposed cysteines may be readily alkylated e.g. a cysteine residue on the β-chains of Mus musculus Hb (Petras & Martin, 1969).

Carbohydrate attachment

Most serum proteins are glycoproteins. Glycosylation occurs as part of the secretion process from the Golgi apparatus of the cell as a classic example of epigenetic change. Of particular relevance to electrophoretic studies is the addition of negatively charged moieties like sialic acid. Sialic acid is invariably terminal in a carbohydrate chain composed of several other hexoses or hexosamines bonded to the protein by a N-glycosyl linkage to an asparagine or glutamine residue. Bonded in this way the sialic acid residue is accessible to neuraminidase excision. Treatment of a glycoprotein with this enzyme often results in a decrease in anodic mobility and a reduction in heterogeneity of the electrophoretic pattern e.g. transferrin and ceruloplasmin (Pantelouris & Arnason, 1967). It was noted by these authors that extracellular proteins generally were glycoproteins, possibly related to their passage through membranes and their recognition by self as "self" during circulation in the vascular system. In contrast, the

intrinsically intra-cellular proteins, especially many enzymes, were not glycoproteins.

Sialization is a specific biosynthetic reaction catalysed by sializing enzymes encoded at distinct loci. A slow phenotype of alkaline phosphatase (a glycoprotein) of the domestic fowl was considered to be the result of a genetic deficiency in the sializing enzyme (Law, 1967). The alkaline phosphatase phenotype possessed no sialic acid and therefore showed slower mobility than the normal phenotype. Foetal intestinal type alkaline phosphatase from human could be distinguished from adult enzyme by greater anodal electrophoretic mobility and its retardation after neuraminidase treatment in vitro, (Mulivor et al., 1978). The authors were uncertain whether the difference in the number of sialic acid residues on the foetal enzyme was a difference in post-translational modification or whether specific genetic loci were involved. Differences in glycoproteins may be due to differences in the carbohydrate chain or may be due entirely to residue substitutions independent of the carbohydrate. Yoshida & Mega, (1979) found that seven variants of human α-1 protease inhibitor possessed the same mannose, galactose, glucosamine and acetyl neuraminic acid contents. The migration differences between these variants was due only to amino acid substitutions not to carbohydrate compositions, regarding which, several laboratories were in disagreement. This work indicates that the variants are alleles of one locus for α-1 protease inhibitor and that the same epigenetic modification affects each variant in the same way. Phenotypes of this

glycoprotein were not due to differences in post-translational modification.

A similar result has been reported for the bovine serum transferrin phenotypes AA, D_1D_1, D_2D_2 and EE by Hatton et al., (1977). Analysis of the purified phenotypes showed the same carbohydrate analyses and the underlying heterogeneity was accounted for in the polypeptide residues.

In the case of bovine β-lactoglobulin the reverse is the case. The β-lactoglobulin Droughtmaster variant (McKenzie, 1967) has the same amino acid composition as the A variant of the protein but a different electrophoretic mobility. The Droughtmaster variant differs in having 5 glucosamine residues plus a sialic acid residue covalently linked to the protein. The other β-lactoglobulin variants A, B and C differ by one or more amino acid residues, but the Droughtmaster variant represents genetic polymorphism in a distinct locus governing carbohydrate attachment but directly affecting the β-lactoglobulin locus product.

Coenzyme and buffer ion binding.

Mixing experiments in which NAD was added to preparations of Drosophila alcohol dehydrogenase (ADH) (Ursprung & Carlin, 1968) appeared to result in shifts in the staining intensity of various bands following electrophoresis. This generation of hybrids in vitro was considered to be caused by the binding of different amounts of coenzyme by the various isoenzymes. Two bands disappeared after dialysis of the impure preparations with 5 mM NAD and two new bands appeared which were not detectable in the original preparation. Cederbaum & Yoshida (1976) reported a complex

series of bands of Rainbow trout G6PD activity after electrophoresis. They conclude that a post-translational change affected NADP binding, causing different amounts of coenzyme to be bound, with ensueing effects on the migration patterns on gels. In a similar manner, Nierthammer & Huennekens (1971) found variable binding of NADP caused polymorphism of human methaemoglobin reductase.

Buffer specific effects can occasionally give rise to more than one zone when a protein is subjected to electrophoresis. Bovine serum albumin gave a single zone on cellulose acetate electrophoresis in phosphate buffer, yet two zones were observed when electrophoresis was carried out in borate buffer. The new band was attributed to the reversible binding of borate anion by the protein (Cann & Goad, 1968).

Proteolytic cleavage.

Many examples are to be found of epigenetic cleavage of proteins as a biosynthetic mechanism <u>in vivo</u>. Proteases themselves are synthesised and stored in an inactive zymogen form e.g. trypsinogen. In a similar manner proteins with such diverse effects as prothrombin and proinsulin are both activated by cleavage of part of their polypeptide chain. In the blood clotting mechanism a series of protein factors with proteolytic activity act one upon the next in a so-called "cascade" sequence. The end result is the formation of fibrin from its precursor, fibrinogen.

Multiple forms of yeast hexokinase were reported to result from the presence of traces of protease activity in the preparation, (Gazith et al., 1968). Two molecular forms of the enzyme were found to be converted to two new, more acidic forms during purification. The conversion occurred with full retention of the enzymes' activities. The original forms were tetrameric whereas the modified enzymes showed a reduced tendency to form tetramers but bound glucose substrate more strongly. Digestive enzymes and certain peptidases may be activated by the injury or disruption of cells, for example during homogenization and extraction, leading to proteolytic modifications and altered phenotypes of the affected proteins.

Miscellaneous modifications.

The phosphorylation or sulphation of certain residues such as tyrosine, serine or threonine is known. The addition of these negatively charged ions will cause faster anodic mobility of the modified proteins on gels. Chymotrypsin and a large group of esterases and proteases possess a serine residue which can be phosphorylated. This reactive serine is also vulnerable to many addition reagents in vitro e.g. diisopropyl fluorophosphate. Addition of such an anionic group would increase anodic mobility of an affected protein. The heterogeneity of ovalbumin from chicken was reduced by treatment of this protein with both acid and alkaline phosphatases to remove one or more phosphate groups (Lush, 1964).

Greater anodal mobility of MDH_m from chicken resulted following iodination of a tyrosine residue (Kaplan, 1968).

This iodination caused helix disruption in the protein and the formation of a new conformer in which more charged residues were exposed, accounting for the faster anodic mobility. Soluble MDH and chicken muscle aldolase were not affected in this way.

Epigenetic change and the Calcutta-1 phenotype.

Many of the examples of post-translational change discussed above were reported to affect the electrophoretic mobility of the modified proteins in such a way as to cause increased anodal bands. Is it possible that the Calcutta-1 phenotype, with its fast anodic appearance, could be explained by one of the known epigenetic changes?

All vertebrate LDH's examined are blocked at the N-terminus by acetylation, (Dayhoff, 1972). Calcutta-1 heterozygote LD_1 and normal LD_1 are no exceptions. No α-amino group was detected by the very sensitive method of Chang (1977) in either of these purified protein preparations - consistent with N-terminal blocking. Specific modifications of lysines and histidines which were stable to the processes of protein digestion and amino acid analysis as detailed in Chapter 7 should have been detected as minor peaks by the Beckman analyser adjacent to the usual lysine and histidine peaks. No evidence was found of such peaks.

Human LD_1 has been shown by the present work to contain probably 5 cysteine residues. The X-ray structures of the two LDH's solved, show no evidence of disulphide bridges. Provided human LDH fits in with the pattern of tertiary

structure already determined for vertebrate LDH from other species (Adams, et al., 1970) each of these sulphydryl residues will be in the reduced form, cysteine. One cysteine near the active site is essential to enzyme activity - Section 7.6. It has become common practice to include 1 mM 2-ME or 1 mM DTT (Pesce et al., 1967; Nadal-Ginard & Markert, 1975) at all stages of LDH preparation to keep the essential cysteine in the reduced form. Activity studies showed that purified enzyme lost activity over a period when 2-ME or DTT was not added. Up to 2 mM DTT was added to enzyme preparations during the heat denaturation experiments in this work (Section 4.2.1) and up to 5 mM DTT during the hybridization studies, Jaenicke et al., (1971). The use of these concentrations of sulphydryl reagent never resulted in either altered absolute mobility on gels or of the relative mobility of the normal and Calcutta bands. Calcutta always appeared as having bands of faster anodal mobility in freshly lysed cells, in all stages of enzyme purification and after months of storage at 4° as the ammonium sulphate precipitate. The two fastest bands in Calcutta LD_1 heterozygote tended to be labile - Figure 3.6.b, which shows only the 3 slowest bands of activity of the 5 bands seen in Figure 3.4. While alkylation of a Calcutta cysteine cannot be ruled out and might well be further investigated, such a gross tertiary change as disulphide bridging in the variant is probably unlikely since it would be expected to affect the circular dichroism of the protein.

Could Calcutta-1 be a glycoprotein? No vertebrate LDH has been shown to be a glycoprotein despite the many

detailed studies of the enzyme from a wide variety of organisms. The X-ray diffraction studies show no carbohydrate residues in the structure. No evidence of amino sugars was obtained in the amino acid analyses. Amino sugars are eluted as a distinct peak in the region of the lysine and histidine peaks from the Beckman analyser (Chapter 7).

Both oxidised and reduced NAD have been used in the Blue Dextran affinity stage of the enzyme purification, to elute the isoenzymes. These eluted fractions containing NAD, both normal and variant isoenzymes were concentrated and routinely subjected to starch gel electrophoresis to monitor isoenzyme separation. Coenzyme was added to the isoenzyme mixtures during hydridization experiments to help preserve enzyme activity. The extent of hybridization was examined by gel electrophoresis. No evidence of shifts in staining intensity or extra bands attributable to differential NAD binding was observed. In respect of buffer ion binding, the Calcutta phenotype was observed unchanged when phosphate, phosphate/citrate, tris/phosphate, tris/HCl or when tris/EDTA/ borate buffers were used for gel electrophoresis, (Appendix-1). The binding of a borate anion causing an extra band as was found in the case of bovine serum albumin, could not explain the fast anodal bands of Calcutta.

The proteolytic cleavage of a significant portion of the Calcutta polypeptide chain would result in a lower subunit molecular weight and possibly in impaired ability to form tetramers with normal subunits. A significantly smaller Calcutta polypeptide is not consistent with the results

obtained (Table 3.1). Similarly protease excision of a small length of peptide is inconsistent with the fingerprints obtained. If excision had occurred as an epigenetic change in Calcutta, one or more peptide spots would be absent or would be of changed mobility as well as fewer spots on the peptide maps.

Epigenetic changes to particular side chains in the Calcutta sequence may be possible. Detailed analysis of individual Calcutta peptides might be used in future experiments to investigate this possibility. Again it is conceivable that epigenetic modification made possible by a genetic mutation e.g. an amino acid substitution followed by a sialic acid attachment might have occurred in the Calcutta variant. I believe however that the existing population genetics and family studies have established beyond reasonable doubt that Calcutta-1 is in fact a genetic mutation and should be regarded as an allele of an LDH locus. For this reason the extremely limited amount of material available has been primarily used in the attempt to identify an amino acid substitution by the peptide mapping technique. Charged residue substitutions have been the most frequent cause of electrophoretic variants and mapping has been pursued as the most likely technique to identify the precise nature of the Calcutta difference.

Since the bulk of the work described in this thesis was completed, Voogt & Khan reported to the International Congress of Genetics in Moscow, 1978, (Voogt & Khan, 1978) that Calcutta-1 is a B subunit mutation. They were able to show by somatic cell fusion and hybridisation techniques of human white cells with rabbit cells, that the Calcutta-1 character resided on chromosome 12. Boone et al. 1972, have already determined that LDH_A locus is on chromosome 11 and LDH_B on chromosome 12. Following this independent evidence for a Calcutta-1 B mutation my further experimental work has focused on demonstrating the structural alteration in Calcutta-1 LD_1.

8.2 Thermal and kinetic properties of Calcutta-1

Experiments designed to examine differences in biochemical properties between LDH's from normal, heterozygous and homozygous Calcutta-1 individuals used several different preparations: haemolysates prepared from whole red cells; purified preparations of the LD_1 isoenzyme from red cells and of LD_5 isoenzyme from extracts of human placenta.

Figures 4.8a, 4.10 clearly show that Calcutta-homozygote LDH is less thermo-stable than normal LDH. The distribution of LDH activity amongst the LD_1, LD_2 and LD_3 bands in Calcutta-homozygote is broadly similar to that of normal (Figure 4.8.b). Haemolysate contains approximately 40% LD_1 (Section 2.1), possibly 35% LD_2 and possibly 20% LD_3 (Figure 4.8.a). It has been demonstrated also (Figure 4.8.a) that the human isoenzymes comply with the order of

thermostability of LDH of other mammals ...

$$LD_5 < LD_4 < LD_3 < LD_2 < LD_1.$$

In the study of total Calcutta-1 homozygote LDH, total activity declined to 11% of the initial activity after 10 min at 70°C while normal declined to about 30% (Figure 4.8.b, 4.10). At 60°C, the respective activities were 57% and 90%. Such a dramatic loss in activity cannot be accounted for by inactivation of the less stable LD_2 and LD_3 bands alone. The levels of the normal activity at 30% after the denaturation (10 min at 70°C) suggest that three-quarters of LD_1 normal remains active, assuming all normal LD_2 and LD_3 are inactivated. A decline to 11% in the same time, however, indicates that some components at least, of the variant LD_1 must be less heat stable than normal.

Heterozygote Calcutta haemolysate behaved in a most interesting way in heat stability studies. The heterozygote enzyme lost activity at almost exactly the same rate (Figure 4.10) and from the same isoenzyme bands (Figure 4.9) as did the normal control. Anodically fast components of the variant LD_1 were still present after 40 min at 60°C (Figure 4.9.b) indicating that the decline in activity was not at the selective expense of mutant components. This result was confirmed by the thermostability studies of purified heterozygote Calcutta LD_1 compared with purified normal LD_1 at 70°C (Figure 4.12) when both activities declined in parallel.

Thermal denaturation of normal haemolysate (Figure 4.8.a) demonstrated that the A subunit-rich isoenzymes were rapidly inactivated at elevated temperatures. In Figure 4.9 no discernible differences in the rates of disappearance of the A subunit-rich isoenzymes of Calcutta-heterozygote and normal haemolysates were observed. Additionally, when the purified LD_5 proteins from variant and normal placentae were subjected to heat denaturation at 50°C (Figure 4.11) activity was lost at comparable rates.

When the thermal kinetics of Calcutta LDH were examined by simple plots of reaction velocity against temperature, curves for normal and variant haemolysate and for normal and variant LD_1 were nearly coincident at various pyruvate concentrations. Plots of normal and variant LD_5 at pyruvate concentrations between 1.5 Km and 4.5 Km (pyruvate) - Figure 4.1 agreed between 20° and 40°C with some divergence at 50°C. This apparent lower stability of Calcutta LD_5 at 50°C was not confirmed by the denaturation experiment.

The Arrhenius plots of Calcutta-homozygote haemolysate and normal haemolysate (Figure 4.5) demonstrate marked differences. Protein denaturation begins between 40°C and 50°C denoted by the concave down plot for Calcutta-homozygote and less efficient catalysis is suggested by the 41% higher Ea value than for normal (Table 4.1). In contrast the catalytic efficiencies of each LD_5 isoenzyme appear to be the same, with parallel Arrhenius plots (Figure 4.7) and activation energy agreeing to 2.8%. The heterozygote purified LD_1 also exhibits the same catalytic efficiency as

normal LD_1 with parallel Arrhenius plots over a revised temperature range (16-44°C) and activation energies agreeing at the 2.5% level. The Km (pyruvate) of the compared LD_1 enzymes increased with temperature in a comparable manner with best agreement being between 30° and 40°C (Figure 4.4). No difference in behaviour of Km (pyruvate) with temperature was seen between normal and Calcutta LD_5, but the Calcutta homozygote haemolysate appeared to be less efficient than normal haemolysate at 50°C - Figure 4.3.

Substrate inhibition for both human LD_1 and human LD_5 was found to be temperature dependent e.g. normal LD_5 - Figure 4.2. Inhibition of normal LD_5 begins at 1 mM pyruvate at 50°C, but at only 0.4 mM at 20°C and at concentrations between these limits at intermediate temperatures. Plots for Calcutta LD_5 gave similar results and have been omitted.

When the catalytic properties of the Calcutta and normal LD_1's were compared, many of the parameters determined were in agreement. Km for pyruvate for the LD_1's and for the haemolysates agreed to within 10% (Section 5.3), the pyruvate optima (Section 5.4) agreed to within 10% (Section 5.3), the pyruvate optima (Section 5.4) agreed at the same level and variation of activity with pH agreed, with optima close to pH 7.5 for the purified LD_1's and pH 7.3 for the haemolysates.

The Km for NADH for the LD_1's and haemolysates show a trend of lowering affinity for coenzyme from the normal to the homozygote Calcutta, with Calcutta heterozygote being intermediate. In contrast to the Km-pyruvate comparisons, Km-NADH might be a more sensitive probe of enzyme conformation,

suggesting an effect at the active site as a result of the Calcutta mutation. The shapes of the pH/activity profiles for purified LD_1's were nearly parallel, declining gently on the acid side and more steeply on the alkaline side. Substrate inhibition occurred over the same concentration range (6 Km-36 Km pyruvate) with the plots for Calcutta-homozygote and normal haemolysate showing some differences. An apparent difference in the extent of inhibition is suggested in Figure 5.1 and the homozygote Calcutta enzyme may be slightly more sensitive to pyruvate inhibition at $30^{\circ}C$.

When the catalytic constants of normal and Calcutta LD_5 are compared, the Km (pyruvate) values differ by 24% (Table 5.2) while the pyruvate optima differ by 11%. Both these determinations agreed at the 10% level for LD_1's. The Lineweaver-Burk plots, at $30^{\circ}C$, of Calcutta and normal LD_5's appeared to be normal and essentially parallel with the 20° and $40^{\circ}C$ regressions (Figure 4.2). The pH profiles of each LD_5 enzyme diverge somewhat (but with greater variation bars) especially at the extremes of pH (Figure 5.4). The pH optima agreed within 3% compared to within 0.5% for the LD_1's. A similar gentle decline in activity between pH 6.5 and pH 4.7 was observed, while from pH 6.5 to pH 8.0 the decline in activity of normal was steeper than for Calcutta. LDH's are acid tolerant in their activities. Activity is still 80% of optimum, 2 pH units below that value, but activity is between 55% and 75% of optimum only 1 pH unit above the optimum for human LD_5.

Turnover numbers were calculated (Tables 5.1, 5.2) for the purified isoenzymes and were of the same order in the compared isoenzymes, but kp values are not a particularly reliable parameter for such comparisons. Duplicate purifications of the same enzyme from the same source can yield 2-fold differences in kp (Dr. E. Heyde, personal communication). Their calculation involves dividing the V_{max} by very dilute enzyme concentrations ($E \sim 10^{-8}M$). The percentage of catalytically active enzyme molecules in the assayed dilution is always uncertain. As observed by Pesce et al., (1967) the kp of the LD_5 isoenzyme is about twice that of the LD_1 from the same animal, a reflection of the greater efficiency of the LD_5 isoenzyme also confirmed by a lower Ea than LD_1.

In all the foregoing catalytic determinations it will be seen that haemolysate values are intermediate between those of purified LD_1's and LD_5's. If haemolysate LDH is assumed to consist of 80% B subunits and 20% A subunits distributed among the isoenzymes, and the catalytic independence of the subunits (Holbrook, et al. 1975) is accepted, then haemolysate results should lie closer to those of LD_1. This effect is best observed with the Km, pyruvate optimum and pH optimum values (Tables 5.1, 5.2, 5.3). The haemolysate LDH's being unpurified yield less reliable determinations as may also be seen by the lower correlation coefficients (r) than for the purified LD_1's (Table 5.1). The kinetic experiments and catalytic constants determined for purified LD_1's or for the haemolysates do not point to large changes in the catalytic mechanism.

8.3 Structural characteristics of Calcutta-1

When the purified LD_1 of normal erythrocytes was compared with the purified heterozygous LD_1 from Calcutta by using circular dichroism, no evidence of an alteration of secondary structure as a result of the mutation was obtained. The α-helix and β structures of the native proteins were conserved and only a suggestion of an altered conformation in the aromatic side-chains was seen.

Circular dichroism of Calcutta and normal LD_5 suggest that no marked alteration has occurred in the secondary structure as a result of the mutation. The amplitudes of the CD curves in the "side chain" region, 260-290 nm of Calcutta and normal are, however, clearly different (Figure 6.2) with non-overlapping of the variation bars. The amplitude difference of 15-20% throughout this wavelength range could result from fewer aromatic residues being present in the Calcutta subunit.

The amino-acid compositions of normal LD_1 and Calcutta-homozygote LD_1 agreed in their lysine and arginine content. In the acidics, comparison is complicated by non-distinguishing of the amide from the acid residues. The Asx (as the most common residue, was used as the reference, to calculate the number of residues/subunit) result was equated (Table 7.1) and the Glx result was higher in the variant. Several apparent peptide changes on the LD_1 tryptic peptide paper maps were checked by peptide analysis but could not be reconciled with a glutamic acid substitution in the Calcutta variant. A tyrosine containing peptide, Tr 5, present in

normal, was absent from the Calcutta LD_1 maps and other apparent alterations in migration in the crowded region of peptides (of limited migration under the system employed) remain to be further examined.

A nanomole-level micro-method of fingerprinting was used to further investigate Calcutta LD_1 and indicated a likely difference peptide was present which could explain the Calcutta variant mobility.

The peptide mapping of each LD_5 showed a difference in peptides named as 4 and 7. In the position of these two spots on the Calcutta map, "half strength" spots, originating from the normal A polypeptide chains (heterozygous variant) should have been visible. These were not observed, even at the sensitive Fluram staining level. The possibility of a "4 + 7" peptide deletion is not consistent with the amplitude difference in the "side-chain" region in the CD study, since the analyses of the 4 and 7 peptides did not include any aromatic residues. Neither is the deletion explanation supported by the SDS/acrylamide experiment, which indicated, if anything, a marginally larger Calcutta subunit (and no suggestion of a doublet) rather than a smaller, deleted subunit. For these reasons the absence of the tryptic peptides 4 and 7 is better explained by a substitution involving a lysine or arginine residue.

8.4 A or B subunit mutation

A number of analytical approaches have been used in this investigation of the LDH variant Calcutta-1 to elucidate the

nature and effects of the mutational change on its properties. Some of the data is conflicting, some results supporting an A subunit mutation, other data supporting a B mutation. In this section the evidence of each analytical approach will be assessed.

8.4.1 Electrophoretic differences

In the electrophoretograms, anodic components of the Calcutta isoenzyme LD_1 to LD_4 were always observed. Homozygote Calcutta LD_1 migrated in starch gels at pH 7 as a single band aligning with the leading edge of the unresolved heterozygote "band" and considerably in advance of the normal LD_1 band, suggesting that it was β_4 and the broad heterozygote "band" was made up of the other combinations of β and B. SDS-acrylamide gels Figures 3.5.b and 3.5.c were loaded at several concentrations with purified LD_1 from Calcutta heterozygote and normal erythrocytes respectively. The presence of SDS molecules saturating the binding sites on the protein subunits would suppress any possible charge differences. However subunits of altered molecular weight should be resolved by this sieving system. No suggestion of doublets in the Calcutta bands was obvious, which indicated that the subunits present are of the same or very closely similar molecular weight to the normal subunits. This result would be expected if only a small change such as an amino-acid substitution or deletion was involved in the mutation.

The resolution of fast anodic bands in zymograms of purified Calcutta-1 heterozygote LD_1 (Figure 3.5) and the

resolution of two protein bands when this pure variant LD_1 was run on alkaline/urea acrylamide gels (Figure 3.4) compared with only a single protein band for normal LD_1 is evidence for a B subunit mutation. A minimum of 5 focused bands were observed for variant LD_1 compared with a single major band for normal LD_1 when these purified proteins were subjected to isoelectric focusing (Figure 3.6). These results are consistent with the expected fast B-variant pattern introduced in Chapter 1 (Figure 1.3.c sample 1).

Electrophoresis of placental extracts on starch gels indicated that the LD_5 band of Calcutta heterozygote possessed variable mobility dependent on pH. That this variable mobility might be artifactual was suspected when an identical migration to normal LD_5 was observed on one gel - Figure 3.2.b. When all of the electrophoretic experiments are considered the weight of evidence excludes the possibility of an Calcutta-1 A subunit charge mutation and indicates a B subunit mutation.

Conclusions. If an A subunit alone was involved in the Calcutta-1 mutation there should be no detectable differences in the electrophoretic behaviour of LD_1. Conversely a B subunit mutation alone should lead to no differences in the electrophoretic behaviour of LD_5. The present experiments indicate that extra mutant bands are present at LD_1 and that only B subunits are involved.

The resolution of Calcutta-1 heterozygote LD_1 into multiple bands by isoelectric focusing was considered the most important result of the electrophoretic experiments.

The next most significant result was the separation of Calcutta LD_1 into only two protein bands, a normal B band and a faster β band, on alkaline urea denaturing gels.

8.4.2 Thermal and kinetic differences

Whole haemolysate thermostability experiments have shown that differential loss of activity in the Calcutta-1 homozygote LDH occurs when compared to normal, which must involve the B subunit. Heterozygote haemolysate LDH is no less heat stable than the normal, suggesting that the presence of the normal B subunits stabilizes the mutant subunits against thermal inactivation. These thermal studies, together with the results of studies on the purified LD_5 isoenzymes from mutant and normal sources, which showed no difference in thermostability, provide further evidence for a B subunit mutation.

Kinetic parameters determined do not appear to be affected by the Calcutta-1 mutation when the heterozygote purified LD_1 and LD_5 were compared with the appropriate normal controls. Similar values were obtained in these comparisons for activation energy, Km (pyruvate), pyruvate optimum, turnover numbers, pH optima, although a difference in Km (pyruvate) for variant and normal LD_5 of 25% was observed. The Arrhenius plots for the purified isoenzymes were linear, in contrast with the plot for Calcutta-homozygote which was non-linear. The lower thermostability of the homozygote variant appears to affect its catalytic efficiency in the higher region of the temperature range studied. A suggestion

of increased sensitivity to high substrate concentration and a higher Km (pyruvate) at 50°C were also noted in the homozygote Calcutta LDH. About 50% higher Km NADH was measured for the homozygote Calcutta LDH and about 20% higher for the heterozygote LDH, than for the normal haemolysate. This trend was confirmed when purified normal and Calcutta heterozygote LD_1 Km's NADH were compared. The results suggest that a conformational change may have occurred in the coenzyme cleft of the mutant subunit.

Conclusions.

Thermo-instability in Calcutta homozygote LDH was considered the most important result from the thermostability experiments. A broken Arrhenius plot for the variant homozygote enzyme, compared with straight plots for heterozygote and normal LD_1's was considered an important follow-up experiment confirming the thermo-instability of the β subunit.

Comparison of kinetic parameters did not high-light large differences in kinetic behaviour of the normal and variant LDH's.

8.4.3 Structural differences and conclusions

The evidence from CD studies, however, is suggestive of A subunit mutation, seen as a change in amplitude of the CD plot in the near ultraviolet. Over the same range of wave-length, plots for Calcutta LD_1 were about 6-10% different, whereas LD_5's were up to 20% different. The CD studies, while useful as comparative experiments of secondary structure

of a gross nature, were not considered to be highly significant in the elucidation of the Calcutta mutant.

Peptide mapping on paper and amino-acid analysis have suggested that differences occur on both the Calcutta LD_1 map and the LD_5 map. For instance for Calcutta LD_1 no peptide Tr 5 containing tyrosine was found, yet this peptide was clearly present in normal LD_1. Peptides Tr 2 and Tr 3 were present on the Calcutta map but absent on the normal map. Peptides Tr 4 and Tr 7 were observed and analysed from the normal LD_5 map but were absent on the Calcutta LD_5 map. This last absence was best explained by an amino-acid substitution of a lysine or arginine.

While some evidence from the structural work suggests a change has occurred in the A subunit, the peptide mapping has reached the stage however, where a B substitution is strongly implied. A consistent change on the tryptic fingerprints (micro-method), of Calcutta LD_1 from two purifications and several mappings was considered a result ranking secondary only to the results of the two electrophoretic experiments referred to in Section 8.4.1.

The evidence of molecular weight estimation, amino-acid analyses, the overall comparability of the peptide maps together with the structural determinations by circular dichroism suggest that subunits of very similar size, primary sequence and tertiary structure are conserved in the Calcutta mutation. Although it was not identified, a charged amino-acid substitution best explains the results obtained in the present work.

Taking all evidence into consideration it is concluded that an amino-acid substitution in the B subunit is the most likely explanation for the Calcutta-1, fast anodic mutation.

Future experiments.

The elucidation of the composition and sequence of the extra Calcutta LD_1 peptide and its relationship to the "sister" peptide are important future experiments. Future peptide analysis is dependent on adequate supplies of purified Calcutta LD_1. LD_1 digestion by other cleaving agents such as CNBr, chymotrypsin and thermolysin could establish by overlap techniques the position of the affected peptide in the overall sequence and by comparison with the pig heart LD_1 X-ray structure, contribute to an understanding of the variant's thermolability.

Further experiments on coenzyme cleft conformation, by the use of coenzyme structural analogues as probes of coenzyme binding, might be informative.

Calcutta-1 in perspective

The extent to which the human organism may tolerate an LDH mutation may be gauged by the finding of a Japanese man deficient in LDH B gene expression (Kitamura et al., 1971). The propositus expressed only the A gene activity in all his tissues examined, including those normally rich in B gene product. The total loss of the tissue-specific, differential expression of the B gene and hence the tissue-specific spectrum of isoenzymes has been successfully overcome by A gene

expression alone in all tissues. By contrast, possession of the LDH Calcutta-1 character represents a very small change in the LDH status of an individual.

8.5 Possible mutation sites

A highly evolved "sophisticated" glycolytic, multisubunit enzyme like LDH appears to be more conservative by a factor of 5 with respect to residue substitutions compared with the more rapidly evolving proteins such as carbonic anhydrase and haemoglobin (Wilson et al., 1977). A consideration of the functional constraints on an enzyme will indicate which regions of the subunit are unlikely to "accept" radical substitutions.

The 20-25% of the LDH subunit in the β sheet contains a high concentration of valines, isoleucines and threonines as well as some 37 charged residues (Holbrook et al., 1975) closely packed into an array which has been termed a "molecule crystal" by Chothia, (1975). This β core is unlikely to accept charged substitutions which cannot be neutralized (Perutz et al., 1968) or insertions or deletions of different amino-acids (Rossmann et al., 1975). In the active site of Dogfish muscle LD_5, many of the residues which interact with, and locate, the NAD coenzyme, and the substrate, have been identified. These include ten charged residues; a further four charged residues take part in the "charged relay" system of the catalytic mechanism.

The subunit binding sites which mutally interact across the three symmetry axes of the regular tetrahedron are

essentially hydrophobic in character (Chothia, 1975; Kauzmann, 1959). Some 35 polar residues are involved in these contacts, many in H-bond interactions, others in ion-pair bonds. Absolute complementarity, whereby subunits "recognize" each other is essential to the maintenance of these short-range interactions (Chothia and Janin, 1975).

Charge substitutions, like Calcutta-1 are unlikely to occur in the regions of the subunit discussed above. In the regions distant from the active site and the subunit binding sites on the subunit surface, charged residues may interact directly with the surrounding aqueous solvent. Substitutions in these surface locations are likely to have the minimal effects on kinetic and thermal properties (homozygote variant excepted) observed for Calcutta-1. It has been argued Zuckerkandl (1976), that a protein fluctuates around a sub-optimal state with respect to solubility and charge. A continuous optimisation process results in an endless series of residue fixations even in a constant environment, which he termed "evolutionary noise". Calcutta-1 might represent just such a fixation.

APPENDIX-1

Starch gel buffers

1. Blake & Kirk (1969)

Gel buffer	0.01M Na_2HPO_4 adjusted to pH 7.0 with citric acid.
Electrode buffer	0.20M Na_2HPO_4/citric acid pH 7.0

2. tris/EDTA/borate (TEB) system of Smithies (1959)

Electrode buffer	0.90M tris, 0.02M EDTA (acid form), 0.50M boric acid pH 8.6.
Gel buffer	$\frac{1}{20}$ dilution of electrode buffer.

3. McIlvaine (1921) wide range citrate-phosphate buffer

Electrode buffer	0.1M citric acid, 0.2M Na_2HPO_4 proportion varied to give final pH in range pH 4.0-pH 7.0.
Gel buffer	$\frac{1}{20}$ dilution of electrode buffer

4. Carter *et al.* (1968)

Electrode buffer	0.1M tris adjusted to pH 8.0 or pH 9.0 with 0.1M NaH_2PO_4.
Gel buffer	$\frac{1}{20}$ dilution of electrode buffer.

5. McKenzie and Treacy (1973) tris-glycine-urea system pH 8.8

Electrode buffer	0.1M tris, 0.19M glycine, pH 8.8
Gel buffer	0.1M tris, 0.19M glycine, 7M urea pH 8.8

6. Bell (1966) formate-urea system pH 3.7

Electrode buffer	0.158M HCO_2H, 0.08M NaOH pH 3.7
Gel buffer	0.052M HCO_2H, 0.0104M NaOH, 8M urea pH 3.0

APPENDIX-2

Acrylamide gel buffers

1. 12½% acrylamide (AM-9), 0.1% SDS

Electrode buffer	0.01M tris, 0.08M glycine, 0.1% SDS pH 8.9
Gel buffer	0.30M tris, 0.048M HCl, 0.1% SDS pH 8.9

2. 5% or 7½% acrylamide (AM-9)

Electrode buffer	0.01M tris, 0.08M glycine pH 8.9
Gel buffer	0.30M tris, 0.048M HCl pH 8.9

3. 4% acrylamide, 8M urea

Electrode buffer	0.06M tris, 0.007M EDTA.Na_2, 0.078M boric acid pH 8.2
Gel buffer	0.40M tris, 0.022M EDTA Na_2, 0.518M boric acid, 8M urea pH 8.7

4. 10% acrylamide, 8M urea Howard and Traut (1973)

Electrode buffer	0.187M glycine, 0.026M HOAc pH 4.0
Gel buffer	0.927M HOAc, 0.048M KOH pH 4.5

APPENDIX - 3

```
@FLIST,F AECMAIN.
UOM FILE LISTER ANU-2      01/25/78    15:04:39
END FLIST  3 CARDS GENERATED.

@HDG,P  ******  PF  ******

@ELT,L  AECMAIN.PF
ELTOT?  RLI870  01/25-15:04:44-(2,)
000001        000             DIMENSION X(20),Y(20),SD(20),A(20),TITLE(14)
000002        000             SUMX=0.0
000003        000             SUMY=0.0
000004        000             SUMXY=0.0
000005        000             SUMX2=0.0
000006        000             SUMY2=0.0
000007        000             READ 7, TITLE
000008        000       7     FORMAT(13A6,A2)
000009        000             PRINT 8, TITLE
000010        000       8      FORMAT(1HO, 'EXPERIMENT NO. = ',13A6,A2//)
000011        000             READ 1,NPTS,NTERMS,MODE
000012        000       1      FORMAT(3I2)
000013        000             DO 10 I=1,NPTS
000014        000             READ 2, X(I),Y(I),SD(I)
000015        000             PRINT 13, X(I),Y(I),SD(I)
000016        000       2     FORMAT(3F10.0)
000017        000      13     FORMAT(1X,F7.3,2F10.5)
000018        000             IF(NTERMS.EQ.3) GO TO 10
000019        000             X(I)=1./X(I)
000020        000             Y(I)=1./Y(I)
000021        000             SUMX=SUMX+X(I)
000022        000             SUMY=SUMY+Y(I)
000023        000             SUMXY=SUMXY+X(I)*Y(I)
000024        000             SUMX2=SUMX2+X(I)*X(I)
000025        000             SUMY2=SUMY2+Y(I)*Y(I)
000026        000      10      CONTINUE
000027        000             CALL POLFIT(X,Y,SD,NPTS,NTERMS,MODE,A,CHISQR)
000028        000             IF(NTERMS.EQ.2) GO TO 5
000029        000             PRINT 3, (A(I),I=1,NTERMS)
000030        000       3     FORMAT(//1HO,'A= ',F10.5,5X,'B = ',F10.5,5X,'C = ',F10.5//)
000031        000             AMAX=-A(2)/(2.*A(3))
000032        000             PRINT 9, AMAX
000033        000       9     FORMAT(1HO,5X,'MAXIMUM = ',FP.3/)
000034        000             GO TO 6
000035        000       5     PRINT 12, (A(I),I=1,NTERMS)
000036        000      12      FORMAT(//1HO,'A = ',F10.5,5X,'B = ',F10.5//)
000037        000             VMAX=1./A(1)
000038        000             XKM=A(2)/A(1)
000039        000             PRINT 11, VMAX,XKM
000040        000      11      FORMAT(1HO,2X,'VMAX = ',F10.4,10X,'KM = ',F10.4//)
000041        000             NCD=(SUMXY-(SUMX*SUMY)/NPTS)**2
000042        000             DCD=(SUMX2-(SUMX**2)/NPTS)*(SUMY2-(SUMY**2)/NPTS)
000043        000             CD= NCD/DCD
000044        000             R= SQRT(CD)
000045        000             PRINT 14, R
000046        000      14     FORMAT(1HO, 'R = ',F10.4//)
000047        000       6     PRINT 4, CHISQR
000048        000       4     FORMAT(1HO,'CHI-SQUARE = ',F10.4)
000049        000             STOP


 ******  PF  ******

000050        000             END

END ELT.

@HDG,N

@FLIST,E E8*ANULIB2.POLFIT
UOM FILE LISTER ANU-2      01/25/78    15:05:25
END FLIST  0 CARDS GENERATED.

@END
@END IGNORED - IN CONTROL MODE

@RESUME,M
```

APPENDIX - 3

```
RLK209*LIBRARY(1),POLFIT
      1         C  SUBROUTINE POLFIT
      2         C
      3         C  PURPOSE
      4         C   MAKE A LEAST-SQUARES FIT TO DATA WITH A POLYNOMIAL CURVE
      5         C       Y=A(1)+A(2)*X+A(3)*X**2+A(4)*X**3+....
      6         C
      7         C  USAGE
      8         C       CALL POLFIT (X,Y,SIGMAY,NPTS,NTERMS,MODE,A,CHISQR)
      9         C
     10         C  DESCRIPTION OF PARAMETERS.
     11         C       X         -ARRAY OF DATA POINTS FOR INDEPENDENT VARIABLE.
     12         C       Y         -ARRAY OF DATA POINTS FOR DEPENDENT VARIABLE.
     13         C       SIGMAY    -ARRAY OF STANDARD DEVIATIONS FOR Y DATA POINTS.
     14         C       NPTS      -NUMBER OF PAIRS OF DATA POINTS.
     15         C       NTERMS    -NUMBER OF COEFFICIENTS (DEGREE OF POLYNOMIAL +1)
     16         C       MODE      -DETERMINES METHOD OF WEIGHTING LEAST-SQUARES FIT
     17         C                   +1 (INSTRUMENTAL) WEIGHT(I)=1.0/SIGMAY(I)**2
     18         C                    0 (NO WEIGHTING) WEIGHT(I)=1.0
     19         C                   -1 (STATISTICAL) WEIGHT(I)=1.0/Y(I)
     20         C       A         -ARRAY OF COEFFICIENTS OF POLYNOMIAL.
     21         C        CHISQR   - UNREDUCED CHI SQUARE FOR FIT.
     22         C
     23         C       SUBROUTINES AND FUNCTION SUBPROGRAMS REQUIRED.
     24         C         DETERM(ARRAY,NORDER)
     25         C                   EVALUATES THE DETERMINANT OF A SYMMETRIC
     26         C                   MATRIX OF ORDER NORDER.
     27         C
     28         C       MODIFICATIONS FOR FORTRAN II
     29         C       OMIT DOUBLE PRECISION SPECIFICATIONS.
     30         C
     31         C       DIMENSION STATEMENT VALID FOR NTERMS UP TO 10.
     32              SUBROUTINE POLFIT (X,Y,SIGMAY,NPTS,NTERMS,MODE,A,CHISQR)
     33              DOUBLE PRECISION SUMX,SUMY,XTERM,YTERM,ARRAY,CHISQ
     34              DIMENSION X(20),Y(20),SIGMAY(20),A(20)
     35              DIMENSION SUMX(19),SUMY(19),ARRAY(10,10)
     36         C
     37         C       ACCUMULATE WEIGHTED SUMS.
     38         C
     39              11 NMAX=2*NTERMS-1
     40                 DO 13 N= 1,NMAX
     41              13 SUMX(N)=0.0
     42                 DO 15 J=1,NTERMS
     43              15 SUMY(J)=0.0
     44                 CHISQ=0.0
     45              21 DO 50 I=1,NPTS
     46                 XI=X(I)
     47                 YI=Y(I)
     48              31 IF (MODE) 32,37,39
     49              32 IF (YI) 35,35,33
     50              33 WEIGHT=1.0/YI
     51                 GO TO 41
     52              35 WEIGHT=1.0/(-YI)
     53                 GO TO 41
     54              37 WEIGHT=1.0
     55                 GO TO 41
     56              39 WEIGHT=1.0/SIGMAY(I)**2
     57              41 XTERM=WEIGHT
     58                 DO 44 N=1,NMAX

     59                 SUMX(N)=SUMX(N)+XTERM
     60              44 XTERM=XTERM*XI
     61              45 YTERM=WEIGHT*YI
     62                 DO 48 J=1,NTERMS
     63                 SUMY(J)=SUMY(J)+YTERM
     64              48 YTERM=YTERM*XI
     65              49 CHISQ=CHISQ+WEIGHT*YI**2
     66              50 CONTINUE
     67         C
     68         C       CONSTRUCT MATRICES AND CALCULATE COEFFICIENTS.
     69              51 DO 54 J=1,NTERMS
     70                 DO 54 K=1,NTERMS
     71                 N=J+K-1
     72              54 ARRAY(J,K)=SUMX(N)
     73                 DELTA=DETERM(ARRAY,NTERMS)
     74                 IF (DELTA) 61,57,61
     75              57 CHISQR=0.0
     76                 DO 59 J=1,NTERMS
     77              59 A(J)=0.0
     78                 GO TO 80
     79              61 DO 72 L=1,NTERMS
     80              62 DO 66 J=1,NTERMS
     81                 DO 65 K=1,NTERMS
     82                 N=J+K-1
     83              65 ARRAY(J,K)=SUMX(N)
     84              66 ARRAY(J,L)=SUMY(J)
     85              70 A(L)=DETERM(ARRAY,NTERMS)/DELTA
     86         C
     87         C       CALCULATE CHI SQUARE
     88         C
     89              71 DO 75 J=1,NTERMS
     90                 CHISQ=CHISQ-2.0*A(J)*SUMY(J)
     91                 DO 75 K=1,NTERMS
     92                 N=J+K-1
     93              75 CHISQ=CHISQ+A(J)*A(K)*SUMX(N)
     94                 CHISQR=CHISQ
     95              80 RETURN
     96                 END

@RESUME,M
```

APPENDIX-4

Treatment of data input for computer program

The standard deviation (S.D.) of each set of velocity readings was calculated manually. There was no reason to expect velocity determinations to be more or less precise at any particular abscissa value (pH or substrate concentration). An overall standardised S.D. was calculated ...

$$s = \sqrt{\frac{(n_1(SD_1)^2 + n_2(SD_2)^2 + \ldots n_j(SD_j)^2)}{N}}$$

where n_j is the number of determinations of velocity at pHj or [pyr]j, SD_j is the standard deviation of this data set and N is the total overall number of determinations for a particular curve.

The computer program was run with all data points entered as input each associated with the standardised S.D. The reason for this treatment of S.D. was to obviate distortions in the computer plotting of curves through particular data sets which by chance had a particularly low SD_j.

APPENDIX-5

Chemical

$NaH_2PO_4.2H_2O$, Na_2HPO_4 (anhydr), $NaHCO_3$, glycine and citric acid were AR grade Univar Chemical, Ajax Chemicals.

Magnesium chloride		J.T.Baker Chemical Co.
Ammonium bicarbonate, ammonium persulphate, sodium chloride, potassium chloride.	AR grade	B.D.H.
Urea	AR grade	Mallinckrodt Chemical Works.
NaOH, KOH		Merke
Ammonium sulphate		May and Baker
Hydrolysed starch		Connaught Medical Laboratories
Acrylamide/bis acrylamide (AM-9)		American Cyanamid Co.
DEAE Sephadex A50, Sepharose 4B, Blue Dextran 2000		Pharmacia
Mercaptoethanol		Eastman Kodak
TEMED		Koch-Light Laboratories
Napthalene Black 10B		Searle Diagnostic
Nigrosine		Matheson, Coleman and Bell

NADH (Grade III) disodium salt, NAD^+ (Grade III), L(+) lactic acid (Grade L-X) lithium salt, sodium pyruvate (Type II), dithiothreitol (D.T.T.), phenazine methosulphate, M.T.T. (tetrazolium bromide), Coomassie brilliant Blue R,

Sigma Chemical Co.

Ninhydrin — Pierce Chemicals

Proteins

Bovine serum albumin	Behringwerke, Marburg-Lahn
B_4 LDH (beef heart, type IX)	Sigma Chemical Co.
A_4 LDH (rabbit muscle, type II)	Sigma Chemical Co.
Trypsin (twice recrystallized)	Worthington Biochemicals
DNAase-1 (crystalline)	Worthington Biochemicals

APPENDIX-6

Circular Dichroism Buffer

0.100 M (Na,K,H) PO_4 pH 7.00 ± 0.01

8.65956 g/l	Na_2HPO_4 (anhydrous)	Mallinckrodt
5.30751 g/l	KH_2PO_4	Mallinckrodt
0.15400 g/l	DTT	Sigma

REFERENCES

Adams, M.J., Ford, G.C., Koekoek, R., Lentz, P.J., McPherson, A. Rossmann,M.G., Smiley, I.E., Schevitz, R.W. and Wonacott, A.J. (1970) Structure of Lactate Dehydrogenase at 2.8 Å resolution. Nature 227 1098-1103

Ananthakrishnan, R., Blake, N.M, Kirk, R.L. and Baxi, A.J. (1970) Further studies on the distribution of genetic variants of Lactate Dehydrogenase in India. Med. J. Aust. 2 787-789

Ananthakrishnan, R. (1972a) Further studies of the distribution of some serum protein and enzyme groups in South India. Humangenetik 15 172-176

Ananthakrishnan, R., Walter, H. and Tsacheva, L. (1972b) Red cell enzyme polymorphisms in Bulgaria. Humangenetik 15 186-190

Apella, E. and Markert, C.L. (1961) Dissociation of Lactate Dehydrogenase into subunits with guanidine hydrochloride. Biochem. biophys. Res. Commun. 6 171-176

Bailey, G.S. and Thye, L.S. (1975) Gene duplication in Salmonid fish: evolution of a Lactate Dehydrogenase with an altered function. In *ISOENZYMES* (Markert, C.L. Ed.) Vol IV pp 401-414 Academic Press, New York.

Baldwin, J. (1973) Lactate Dehydrogenases of monotremes: presence of an additional subunit and absence of LDHX. Proc. Aust. biochem. Soc. 6 70.

Baldwin, J. and Aleksiuk, M. (1973) Adaption of enzymes to temperature: Lactate and Malate Dehydrogenases from Platypus and Echidna. Comp. Biochem. Physiol. 44B 363-370

Baldwin, J. and Temple-Smith, P. (1973) Distribution of LDHX in mammals: presence in marsupials and absence in the monotremes Platypus and Echidna. Comp. Biochem. Physiol. 46B 805-811

Balinsky, D. (1966) Kinetic properties and mechanism of action of human heart Lactate Dehydrogenase and α-Hydroxybutyrate Dehydrogenase. Biochim. biophys. Acta. 122 537-540

Bell, K. (1966) Table I. Summary of zone electrophoretic methods. In *MILK PROTEINS* (McKenzie, H.A. Ed.) Vol. II p.500 Academic Press, New York

Blake, C.C.F. (1974) Evolution of nucleotide-binding proteins. Nature 250 284-285

Blake, N.M., Kirk, R.L., Pryke, E. and Sinnett, P. (1969a) Lactate Dehydrogenase electrophoretic variant in a New Guinea Highland population. Science 163 701-702

Blake, N.M. and Kirk, R.L. (1969b) New genetic variant of 6-Phosphogluconate Dehydrogenase in Australian Aborigines. Nature 221 278

Blake, N.M., Kirk, R.L., and Baxi, A.J. (1970) The distribution of some enzyme group systems among Marathis and Gujaratis in Bombay. Human Hered. 20 409-416

Blake, N.M., Kirk, R.L., McDermid, E.M., Omoto, K. and Ahuja, Y.R. (1971) The distribution of serum protein and enzyme group systems among North Indians. Human Herd. 21 440-457

Blake, N.M., McDermid, E.M., Kirk, R.L., Ong, Y.W., Simons, M.J. (1973a) The distribution of red cell enzyme groups among Chinese and Malays in Singapore. Sing. Med. J. 14 2-8

Blake, N.M., Omoto, K., Kirk, R.L. and Gajdusek, D.C. (1973b) Variation in red cell enzyme groups among populations of the Western Caroline Islands, Micronesia. Am. J. hum. Genet. 25 413-421

Blake, N.M. Kirk, R.L., McDermid, E.M., Case, J. and Bashir, H. (1973c) The distribution of blood, serum protein and enzyme groups in a series of Lebanese in Australia. Aust. J. exp. Biol. med. Sci. 51 209-220

Blanco, A., Zinkham, W.H. (1963) Lactate Dehydrogenases in human testes. Science 139 601-602

Boyer, S.H., Fainer, D.C. and Watson-Williams, E.J. (1963) Lactate Dehydrogenase variant from human blood: evidence for molecular subunits. Science 141 642-643

Brodelius, P. and Mosbach, K. (1973) Separation of the isoenzymes of Lactate Dehydrogenase by affinity chromatography using immobilized AMP-analogue. FEBS Lett. 35 223-226

Buehner, M. Ford, G.C., Moras, D. Olsen, K.W. and Rossmann, M.G. (1973) D-Glyceraldehyde-3-Phosphate Dehydrogenase: three-dimensional structure and evolutionary significance. Proc. natn. Acad. Sci. 70 3052-3054

Cahn, R.D., Kaplan, N.O., Levine, L. and Zwilling, E. (1962) Nature and development of Lactic Dehydrogenases. Science 136 962-969

Campbell, J.W., Watson, H.C. and Hodgson, G.I. (1974) Structure of yeast Phosphoglycerate Mutase. Nature 250 301-303

Carter, N.D., Fildes, R.A., Fitch, L.I. and Parr, C.W. (1968) Genetically determined electrophoretic variations of human Phosphogluconate Dehydrogenase. Acta genet., Basel 18 109-122

Cartwright, R.A. (1976) Unifactorially inherited attributes of the population of Holy Island, Northumberland. Ann. hum. Biol. 3 351-362

Chen, Y-H., and Yang, J.T. (1971) A new approach to the calculation of secondary structures of globular proteins by optical rotatory dispersion and circular dichroism. Biochem. Biophys. Res. Commun. 44 1285-1291

Chen, Y-H., Yang, J.T. and Chau, K.H. (1974) Determination of the helix and β form of proteins in aqueous solution by circular dichroism. Biochemistry 13 3350-3359

Chilson, O.P., Costello, L.A. and Kaplan, N.O. (1964) Hybridization of Lactic Dehydrogenases *in vitro* by concentrated sodium chloride and by reversible inactivation in urea. J. mol. Biol. 10 349-352

Chothia, C. (1975) Structural invariants in protein folding. Nature 254 304-308

Chothia, C. and Janin, J. (1975) Principles of protein-protein recognition. Nature 256 705-708

Clark, P.I., Kostuk, W.J. and Henderson, A.R. (1976) A simple and rapid procedure for the preparation of human LDH-1 and LDH-5 using an ion-exchange mini-column. Clinica chim. Acta 69 361-366

Cuatrecasas, P. and Anfinsen, C.B. (1971) Affinity chromatography In *METHODS IN ENZYMOLOGY* (Colowick, S.P. and Kaplan, N.O. Eds) Vol XXII pp 345-378

Das, S.R., Mukherjee, B.N., Das, S.K., Ananthakrishnan, R., Blake, N.M. and Kirk, R.L. (1970a) LDH variants in India. Humangenetik 9 107-109

Das, S.R., Mukherjee, B.N., Das, S.K., Blake, N.M. and Kirk, R.L. (1970b) The distribution of some enzyme group systems among Bengalis. Indian J. med. Res. 58 866-875

Das, S.R., Mukherjee, B.N. and Das, S.K. (1972a) Caste and age variation of the incidence of LDH variants in the Bengali Hindus. Humangenetik 14 151-154

Das, S.R., Mukherjee, B.N., Das, S.K. and Malhotra, K.C. (1972b) Four types of genetic variant of LDH found in India. Human Hered. 22 264-270

Das, S.R., Malhotra, K.C., Mukherjee, B.N. and Das, S.K. (1973a) Genetic variants of Lactate Dehydrogenase in the Dhangars of Maharashtra, India. Jap. J. human Genet. 18 305-308

Das, S.R., Malhotra, K.C., Mukherjee, B.N. and Das, S.K. (1973b) LDH and MDH variants in five castes around Delhi, India. Technical Report No.151 Anthrop/5/73 Indian Statistical Institute, Calcutta.

Davidson, R.G., Fildes, R.A., Glen-Bott, A.M., Harris, H., Robson, E.B., Cleghorn, T.E. (1965) Genetical studies on a variant of human Lactate Dehydrogenase (subunit A). Ann. hum. Genet. 29 5-17

Dayhoff, M.O. (1972) In *ATLAS OF PROTEIN SEQUENCE AND STRUCTURE* (Dayhoff, M.O. Ed.) Vol 5 pp 1-99 National Medical Research Foundation, Washington.

Dixon, M. and Webb, E.C. (1959) Effect of temperature. In *ENZYMES* pp 150-170 Longmans, Green & Co., London.

Easley, C.W. (1965) Combinations of specific color reactions useful in the peptide mapping technique. Biochim. Biophys. Acta. 107 386-388

Edelhoch, H. and Osborne, J.C. (1976) The thermodynamic basis of the stability of proteins, nucleic acids and membranes. In *ADVANCES IN PROTEIN CHEMISTRY* (Anfinsen, C.B., Edsall, J.T. and Richards, F.M. Eds) Vol 30 pp 183-250 Academic Press, New York.

Elving, P.J., Markowitz, J.M. and Rosenthal, I. (1956) Preparation of buffer systems of constant ionic strength. Analyt. Chem. 28 1179-1180

Eventoff, W., Hackert, M.L., Steindel, S.J., and Rossmann, M.G. (1975) A structural comparison of Porcine B_4 and Dogfish A_4 isozymes of Lactate Dehydrogenase. In *ISOENZYMES* (Markert, C.L. Ed.) Vol I pp 137-150 Academic Press, New York.

Everse, J. and Kaplan, N.O. (1973) Lactate Dehydrogenases: structure and function. Advances in Enzymology 37 61-133

Farhud, D.D., Ananthakrishnan, R., Walter, H., Loser, J. (1973) Electrophoretic investigation of some red cell enzymes in Iran. Human Hered. 23 263-266

Fine, I.H. and Costello, L.A. (1963) The use of starch electrophoresis in Dehydrogenase studies. In *METHODS IN ENZYMOLOGY* (Colowick, S.P. and Kaplan, N.O. Eds) Vol VI pp 958-972 Academic Press, New York.

Fisher, R.A., Putt, W. and Harris, H. (1977) The distirubtion of isoelectric points of human soluble proteins and in particular the enzyme products of 88 human gene loci. Ann. hum. Genet. 40 371-384

Fleisher, J.H. (1957) Thermal denaturation of Lobster Hemocyanin. Biochim. biophys. Acta. 23 28-33

Fondy, T.P., Pesce, A., Freedberg, I., Stolzenbach, F. and Kaplan, N.O. (1964) The comparative enzymology of Lactic Dehydrogenases. II. Properties of the crystalline HM_3 hybrid from Chicken muscle and of H_2M_2 hybrid and H_4 enzyme from Chicken liver. Biochemistry 3 522-530

Fritz, P.J., Morrison, W.J., White, E.L. and Vesell, E.S. (1970) Comparative study of methods for quantitation of Lactate Dehydrogenase isozymes. Analyt. Biochem. 36 443-453

Ganesan, J., Lie-Injo, L.E., Beng, P.O. (1976) Variation of several erythrocyte enzymes in the Dayaks of Sarawak. Human Hered. 26 124-127

Godber, M., Kopeć, A.C., Mourant, A.E., Teesdale, P., Tills, D., Weiner, J.S., El-Niel, H., Wood, C.H., and Barley, S. (1976) The blood groups, serum groups, red-cell isoenzymes and haemoglobins of the Sandawe and Nyaturu of Tanzania. Ann. hum. Biol. 3 463-473

Goldberg, E. (1972) Amino acid composition and properties of crystalline Lactate Dehydrogenase X from mouse testes. J. biol. Chem. 247 2044-2048

Goldberg, E. (1963) Lactic and Malic Dehydrogenases in human spermatozoa. Science 139 602-603

Hamada, M. (1975) Relationship between the change in the tertiary structure of human tumour Lactate Dehydrogenase and carcinogenesis. In *ISOENZYMES* (Markert, C.L. Ed.) Vol II pp 143-156 Academic Press, New York.

Hamilton, H.B., Ueda, N. Kishimoto, S., Satoh, C. (1975) Polymorphisms of erythrocyte enzymes and serum proteins (Report 2). Jap. J. hum. Genet. 20 36-37

Herzog, P., Bohatoua, J., Drdova, A. (1970) Genetic polymorphisms in Kenya. Am. J. hum. Genet. 22 287-291

Hewett-Emmett, D., Cook, C.N. and Barnicot, N.A. (1976) Old World Monkey haemoglobins: deciphering phylogency from complex patterns of molecular evolution. In *MOLECULAR ANTHROPOLOGY* (Goodman, M. and Tashian, R.E. Eds) Plenum Press, New York.

Hill, E., Tsernoglou, D., Webb, L., Banaszak, L.J. (1972) Polypeptide conformation of cytoplasmic Malate Dehydrogenase from an electron density map at 3.0 Å resolution. J. mol. Biol. 72 577-591

Holbrook, J.J., Liljas, A., Steindel, S.J. and Rossmann, M.G. (1975) Lactate Dehydrogenase. In *THE ENZYMES* (Boyer, P.D. Ed.) Vol XI pp 191-292 Academic Press, New York.

Holmes, R.S. (1972) Evolution of Lactate Dehydrogenase genes. FEBS Lett. 28 51-55

Holmes, R.S., Cooper, D.W., and Vandeberg, J.L. (1973) Marsupial and monotreme Lactate Dehydrogenase isoenzymes: phylogeny, ontogeny and homology with eutherian mammals. J. exp. Zool. 184 127-148

Holmes, R.S. and Scopes, R.K. (1974) Immunochemical homologies among vertebrate Lactate Dehydrogenase isozymes. Eur. Jnl. Biochem. 43 167-177

Holzwarth, G. and Doty, P. (1965) The ultraviolet circular dichroism of polypeptides. J. Am. chem. Soc. 87 218-228

Horowitz, J.J. and Whitt, G.S. (1972) Evolution of a nervous system specific Lactate Dehydrogenase isozyme in fish. J. exp. Zool. 180 13-32

Howard, G.A. and Traut, R.R. (1973) Separation and radio-autography of microgram quantities of ribosomal proteins by two-dimensional polyacrylamide gel electrophoresis. FEBS Lett. 29 177-180

Ingram, V.M. (1962) The evolution of a protein. Fedn. Proc. 21 1053-1057

Jaenicke, R., Koberstein, R. and Teuscher, B. (1971) The enzymically active unit of Lactic Dehydrogenase. Molecular properties of Lactic Dehydrogenase at low protein and high salt concentrations. Eur. Jnl. Biochem. 23 150-159

Kalckar, H.M. (1947) Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzyme of purine metabolism. J. biol. Chem. 167 461-475

Kaloustian, H.D. and Kaplan, N.O. (1969) Lactate Dehydrogenase of Lobster (*Homarus americanus*) tail muscle. J. biol. Chem. 244 2902-2910

Kanno (1977) in press. LDH variant Tokyo-1. Personal communication, Omoto, K. (1976)

Kauzmann, W. (1959) Some factors in the interpretation of protein denaturation. In *ADVANCES IN PROTEIN CHEMISTRY* (Anfinsen, C.B., Anson, M.L., Bailey, K. and Edsall, J.T. Eds) Vol XIV pp 1-63 Academic Press, New York.

Kirk, R.L., Blake, N.M. and Vos, G.H. (1971) The distribution of enzyme group systems in a sample of South African Bantu. S. Afr. med. J. 45 69-72

Kirk, R.L., Blake, N.M., Cooke, D.R. and Jose, D.G. (1972) The distribution of some serum protein and enzyme groups among Aboriginal populations in Queensland. Hum. Biol. Oceania 1 207-214

Kirk, R.L. McDermid, E.M., Blake, N.M., Wight, R.L., Yap, E.H., Simons, M.J. (1973a) The distribution of red cell enzyme and serum protein groups in a population of Dani (Pit River, West Irian). Humangenetik 17 345-350

Kirk, R.L. (1973b) Serum protein and enzyme groups in physical anthropology with special reference to Indian populations. In *PHYSICAL ANTHROPOLOGY AND ITS EXTENDING HORIZONS - S.S. SARKAR MEMORIAL VOLUME* (Basu, A., Ghosh, A.K., Biswas, S.K., and Ghosh, R. Eds) pp 189-209 Orient Longman, Calcutta.

Kirk, R.L., McDermid, E.M., Blake, N.M., Gajdusek, D.C., Leyshon, W.C. and MacLennan, R. (1974) Blood group, serum protein and red cell enzyme groups of Amerindian populations in Colombia. Am. J. phys. Anthrop. 41 301-316

Kirk, R.L., Keats, B., Blake, N.M., McDermid, E.M., Ala, F., Karimi, M., Nickbin, B., Shabazi, H. and Kmet, J. (1977) Genes and people in the Caspian Littoral: a population genetic study in Northern Iran. Am. J. phys. Anthrop. 46 377-390

Koen, A.L. and Goodman, M. (1969) Evidence that genes at two loci code for Lactate Dehydrogenase subunit B in *Nycticebus coucang*. Biochem. Genet. 3 595-601

Kraus, A.P. and Neely, C.L. (1964) Human erythrocyte Lactate Dehydrogenase: four genetically determined variants. Science 145 595-597

Levi, A.S. and Kaplan, N.O. (1971) Physical and chemical properties of reversibly inactivated Lactate Dehydrogenases. J. biol. Chem. 246 6409-6417

Lie-Injo, L.E., Lopez, C.G., Ganesan, J. (1973) Lactic Dehydrogenase variants in different racial groups in Malaysia. Human Hered. 23 487-491

Lie-Injo, L.E., Kosasih, E.N., Tann, G. (1974) Variation of several erythrocyte enzymes and serum proteins of Indonesians from North Sumatra. Humangenetik 22 331-334

Malcolm, L.A., Woodfield, D.G., Blake, N.M., Kirk, R.L. and McDermid, E.M. (1972) The distribution of blood, serum protein and enzyme groups on Manus Island (Admiralty Islands, New Guinea). Human Hered. 22 305-322

Marengo-Row, A.J., Aviet, K., Godber, M.J., Kopeć, A.C., Mourant, A.E., Tills, D., and Woodhead, B.J. (1974) The inherited blood factors of the inhabitants of Southern Arabia. Ann. hum. Biol. 1 311-326

Markert, C.L. (1963) Lactate Dehydrogenase isozymes: dissociation and recombination of subunits. Science 140 1329-1330

Markert, C.L. and Appella, E. (1963) Immunochemical properties of Lactate Dehydrogenase isoenzymes. Ann. N.Y. Acad. Sci. 103 913-929

Markert, C.L. and Faulhaber, I. (1965) Lactate Dehydrogenase isozyme patterns of fish. J. exp. Zool. 159 319-332

Markert, C.L. and Massaro, E.J. (1968) Lactate Dehydrogenase isoenzymes: dissociation and denaturation by dilution. Science 162 695-697

Markert, C.L. and Holmes, R.S. (1969) Lactate Dehydrogenase isozymes of the Flatfish, *Pleuronectiformes*: kinetic, molecular and immunochemical analysis. J. exp. Zool. 171 85-104

Markert, C.L., Shaklee, J.B. and Whitt, G.S. (1975) Evolution of a gene. Science 189 102-114

Massaro, E.J. and Markert, C.L. (1968) Isozyme patterns of Salmonid fishes: evidence for multiple cistrons for Lactate Dehydrogenase polypeptides. J. exp. Zool. 168 223-238

Masters, C.J. and Holmes, R.S. (1975) *HAEMOGLOBIN, ISOENZYMES AND TISSUE DIFFERENTIATION* (Neuberger, A. and Tatum, E.L. Eds) Vol 42 *FRONTIERS OF BIOLOGY* pp 23-25 North-Holland, Amsterdam.

McAlpine, P.J., Hopkinson, D.A. and Harris, H. (1970) Thermostability studies on the isoenzymes of human Phosphoglucomutase. Ann. hum. Genet. 34 61-71

McAlpine, P.J., Chen, S.H., Cox, D.W., Dossetor, J.B., Giblett, E., Steinberg, A.G. and Simpson, N.E. (1974) Genetic markers in blood in a Canadian Eskimo population with a comparison of allele frequencies in circumpolar populations. Human Hered. 24 114-142

McDermid, E.M., Blake, N.M., Kirk, R.L., Kosasith, E.N., Simons, M.J. (1973) The distribution of serum protein and enzyme groups among the Batak of Samosir Island (Sumatra, Indonesia). Humangenetik 17 351-356

McIlvaine, T.C. (1921) A buffer solution for colorimetric comparison. J. biol. Chem. 49 183-186

McKenzie, H.A. and Treacy, G.B. (1973) personal communication.

Michl, H. (1958) Hochvoltelektrophorese. J. Chromat. 1 93-121

Millar, D.B., Summers, M.R. and Niziolek, J.A. (1971) Spontaneous *in vitro* hybridization of LDH homopolymers in the undenatured state. Nature: New Biology 230 117-119

Modiano, G., Bernini, L., Carter, N.D., Benerecetti, S.A.S., Detter, J.G., Baur, E.W., Paolucci, A.M., Gigliani, F., Morpurgo, G., Santolamazza, C., Scoazzari, R., Terrenato, L., Khan, P.M., Nijenhuis, L.E., Kanashiro, V.K. (1972) A survey of several red cell and serum genetic markers in a Peruvian population. Am. J. hum. Genet. 24 111-123

Moore, S., Spackman, D.H. and Stein, W.H. (1958) Chromatography of amino acids on sulfonated polystyrene resins. Analyt. Chem. 30 1185-1190

Morrison, W.J. and Wright, J.E. (1966) Genetic analysis of three Lactate Dehydrogenase isoenzyme systems in Trout: evidence for linkage of genes coding subunits A and B. J. exp. Zool. 163 259-270

Motulsky, A.G. (1974) Genetics of human hemoglobins: an overview. Ann. N.Y. Acad. Sci. 241 7-11

Mourant, A.E., Beckman, L., Beckman, G., Nilsson, L.O., and Tills, D. (1968a) Erythrocyte Lactate Dehydrogenase variants in the Icelandic and Swedish populations. Acta genet., Basel 18 553-555

Mourant, A.E., Godber, M.J. and Kopec´ A.C. (1968b) The hereditary blood factors of some populations in Bhutan. In *ANTHROPOLOGIST* (special volume) pp 29-43) University of Delhi.

Mukherjee, B.N., Das, S.K. and Dash Sharma, P., (1973) Study of LDH variants amongst the Mundas in Ranchi District, Bihar, India. Humangenetik 19 285-287

Mukherjee, B.N., Das, S.K., Basak, S.N. and Kellermann, G. (1974) The distribution of some erythrocyte and serum enzyme group systems in the Mahishyas and the Muslims of 24-Parganas, West Bengal. Hum. Biol. 46 425-433

Mukherjee, B.N., Das, S.K. and Ghose, B.R. (1975a) Study of three enzyme group systems: Phosphoglucomutase, Lactate Dehydrogenase and Malate Dehydrogenases in a Punjabi population of Delhi. Indian J. phys. Anthropol. & hum. Genet. 1 67-74

Mukherjee, B.N., Das, S.K., and Dash Sharma, P. (1975b) Serum protein and red cell polymorphisms in Oraon tribe, India. Ann. hum. Biol. 2 201-204

Mukherjee, B.N., Das, S.K., Malhotra, K.C., Kate, S.L., Muthlik, G.S., Sainani, G.S. and Bhidye, S. (1975c) Placental enzyme polymorphisms amongst Maharastrians. A study of Alkaline Phosphatase and Lactate Dehydrogenase. Proc. Indian. Soc. hum. Genet. 2nd Annual Conference.

Nadal-Ginard, B. and Markert, C.L. (1975) Use of affinity chromatography for purification of Lactate Dehydrogenase and for assessing the homology and function of the A and B subunits. In *ISOENZYMES* (Markert, C.L. Ed.) Vol II pp 45-67 Academic Press, New York.

Nakano, E. and Whitely, A.H. (1965) Differentiation of multiple molecular forms of four Dehydrogenases in the teleost *Oryzias latipes*, studied by disc electrophoresis. J. exp. Zool. 159 167-179

Nance, W.E., Claflin, A., Smithies, O. (1963) Lactic Dehydrogenase: genetic control in man. Science 142 1075-1077

Neel, J.V., Ferrell, R.E. and Conrad, R.A. (1976) The frequency of rare protein variants in Marshall Islanders and other Micronesians. Am. J. hum. Genet. 28 262-269

Nilsson, L.O. and Eriksson, A.W. (1972) Screening for haemoglobin and Lactate Dehydrogenase variants in the Icelandic, Swedish, Finnish, Lappish, Mari and Greenland Eskimo populations. Human Hered. 22 372-379

Nisselbaum, J.S. and Bodansky, O. (1961) Purification and properties of human heart Lactic Dehydrogenase. J. biol. Chem. 236 323-327

Nisselbaum, J.S. and Bodansky, O. (1963) Purification, kinetic and immunochemical studies of the major variants of Lactic Dehydrogenase from human liver, hepatoma, and erythrocytes; comparison with the major variant of human heart Lactic Dehydrogenase. J. biol. Chem. 238 969-974

Notstrand, B., Vaara, I. and Kannan, K. (1975) Structural relationship of human erythrocyte Carbonic Anydrase isozymes B and C. In *ISOENZYMES* (Markert, C.L. Ed.) Vol I pp 575-599 Academic Press, New York.

O'Carra, P., and Barry, S. (1972) Affinity chromatography of Lactate Dehydrogenase. Model studies demonstrating the potential of the technique in mechanistic investigation as well as in the purification of multi-substrate enzymes. FEBS Lett. 21 281-285

Odense, P.H., Leung, T.C., Allen, T.M. and Parker, E. (1969) Multiple forms of Lactate Dehydrogenase in the Cod, *Gadus morhua L.* Biochem. Genet. 3 317-334

Ohno, S. (1970) *EVOLUTION BY GENE DUPLICATION* Springer-Verlag, New York.

Okabe, K., Hayakawa, T., Hamada, M. Koike, M. (1968) Purification and comparative properties of human Lactate Dehydrogenase isozymes from uterus, uterine myoma, and cervical cancer. Biochemistry 7 79-90

Omoto, K. and Harada, S. (1972) The Ainu of Hokkaido. J. Fac. Sci., Tokyo Univ. Sec V 171-211 quoted in JIBP *SYNTHESIS* (International Biological Program) 2 Human Adaptability (Watanabe, S., Kondo, S. and Matsunaga, Eds) University of Tokyo Press, Tokyo.

Osborne, W.R.A. and Tashian, R.E. (1974) Thermal inactivation studies of normal and variant human erythrocyte Carbonic Anhydrases by using a sulphonamide-binding assay. Biochem. J. 141 219-255

Papiha, S.S., Roberts, D.F., Ali, S.G.M., Islam, M.M. (1975) Some Hereditary blood factors of the Bengali Muslim of Bangladesh. Red cell enzymes, haemoglobins, and serum proteins). Humangenetik 28 285-293

Perrin, D.D. and Dempsey, B. (1974) In *BUFFERS FOR pH AND METAL ION CONTROL*. (A. Albert, Ed.) Chapman and Hall, London.

Perutz, M.F. and Lehmann, H. (1968) Molecular pathology of human haemoglobin. Nature 219 902-909

Pesce, A., McKay, R.H., Stolzenbach, F., Cahn, R.D. and Kaplan, N.O. (1964) The comparative enzymology of Lactic Dehydrogenases I. Properties of the crystalline Beef and Chicken enzymes. J. biol. Chem. 239 1753-1761

Pesce, A., Fondy, T.P., Stolzenbach, F., Castillo, F. and Kaplan, N.O. (1967) The comparative enzymology of Lactic Dehydrogenases. III. Properties of the H_4 and M_4 enzymes from a number of vertebrates. J. biol. Chem. 242 2151-2167

Plagemann, P.G.W., Gregory, K.F., Wróblewski, F. (1960) The electrophoretically distinct forms of mammalian Lactic Dehydrogenase. I. Distribution of Lactic Dehydrogenase in rabbit and human tissues. J. biol. Chem. 235 2282-2287

Plagemann, P.G.W., Gregory, K.F. and Wróblewski, F. (1961) Die elektrophoretisch trennbaren Lactat-dehydrogenasen des Säugetieres. III. Einfluß der temperatur auf die Lactat-dehydrogenasen des kaninchens. Biochem. Z. 334 37-48

Reys, L., Manso, C., Stamatoyannopoulos, G. and Giblett, E. (1972) Genetic studies on Southeastern Bantu of Mozambique. II. Serum groups, haemoglobins and red cell isozyme phenotypes. Humangenetik 16 227-233

Roberts, D.F., Papiha, S.S. and Abeyaratne, K.P. (1972) Red cell enzyme polymorphisms in Ceylon Sinhalese. Am. J. hum. Genet. 24 181-188

Rossmann, M.G., Adams, M.J., Buehner, M., Ford, G.C., Hackert, M.L., Lentz, P.J., McPherson, A., Schevitz, R.W. and Smiley, I.E. (1971) Structural constraints of possible mechanisms of Lactate Dehydrogenase as shown by high resolution studies of the apoenzyme and a variety of enzyme complexes. Cold Spring Harbour Symp. quant. Biol. 36 179-191

Rossmann, M.G., Moras, D. and Olsen, K.W. (1974) Chemical and biological evolution of a nucleotide-binding protein. Nature 250 194-199

Rossmann, M.G., Liljas, A., Branden, C-I, and Banasak, L.J. (1975) Evolutionary and structural relationships among dehydrogenases. In *THE ENZYMES* (Boyer, P.D. Ed) Vol XI pp 62-102 Academic Press, New York.

Ryan, L.D. and Vestling, C.S. (1974) Rapid purification of Lactate Dehydrogenase from rat liver and hepatoma: a new approach. Archs Biochem. Biophys. 160 279-284

Saha, N., Kirk, R.L., Shanbhag, S., Joshi, S.H. and Bhatia, H.M. (1974a) Genetic studies among the Kadar of Kerala. Human Hered. 24 198-218

Saha, N., Kirk, R.L., Undevia, J.V. (1974b) Two further cases of homozygosity for a Lactate Dehydrogenase variant gene. Am. J. hum. Genet. 26 723-726

Saha, N. Kirk, R.L., Shanbhag, S. Joshi, S.R., Bhatia, H.M. (1976) Population genetic studies in Kerala and the Nilgiris (South West India) Human Hered. 26 175-197

Schneider, P., Anathakrishnan, R., Walter, H., Xirotiris, N. and Abele, R. (1975) Enzyme polymorphisms and haemoglobin variants in Greeks. Humangenetik 27 217-222

Schulz, G.E. and Schirmer, R.H. (1974) Topological comparison of Adenyl Kinase with other proteins. Nature 250 142-144

Segel, I.H. (1975) Behaviour and analysis of rapid equilibrium and steady-state enzyme systems. In *ENZYME KINETICS* pp 44-49 John Wiley & Sons, New York.

Sensabaugh, G.F. and Kaplan, N.O. (1972) A Lactate Dehydrogenase specific to the liver of Gadoid fish. J. biol. Chem. 247 585-593

Shapiro, E., Ben-Yoseph, Y., Eyal, F.G. and Russell, A. (1974) Enzymatically inactive red cell Carbonic Anhydrase B in a family with renal tubular acidosis. J. clin. Invest. 53 59-63

Shaw, C.R. and Barto, E. (1963) Genetic evidence for the subunit structure of Lactate Dehydrogenase isozymes. Proc. natn. Acad. Sci. 50 211-214

Shih, L-Y., Hsia, D.Y-Y. (1969) The distribution of genetic polymorphisms among Chinese in Taiwan. Human. Hered. 19 227-233

Singh, S., Sareen, K.N., and Goedde, H.W. (1974) Investigation of some biochemical genetic markers in four endogamous groups in Panjab (N.W. India). II. Red cell enzyme polymorphisms. Humangenetik 22 133-138

Sinnett, P., Blake, N.M., Kirk, R.L., Lai, L.Y.C. and Walsh, R.J. (1970) Blood, serum protein and enzyme groups among Enga-speaking people of the Western Highlands, New Guinea, with an estimate of genetic distance between clans. Archaeol. & phys. Anthrop. Oceania (Aust.) 5 236-252

Smithies, O. (1959) An improved procedure for starch-gel electrophoresis: further variations in the serum proteins of normal individuals. Biochem. J. 71 585-587

Somero, G.N. and Low, P.S. (1977) Enzyme hydration may explain catalytic efficiency differences among Lactate Dehydrogenase homologues. Nature 266 276-278

Stammers, D.K. and Muirhead, H. (1977) Three-dimensional structure of cat muscle Pyruvate Kinase at 3.1 Å resolution. J. mol. Biol. 112 309-316

Straus, J.H., Gordon, A.S. and Wallach, D.F.H. (1969) The influence of tertiary structure upon the optical activity of three globular proteins: myoglobin, hemoglobin and lysozyme. Eur. Jnl. Biochem. 11 201-212

Su, Y.Y.T. and Jirgensons, B. (1977) Further studies on detergent-induced conformational transitions in proteins. Circular dichroism of Ovalbumin, bacterial α-Amylase, Papain, and β-Lactoglobulin at various pH values. Archs Biochem. Biophys. 181 137-146

Südi, J. (1970) Temperature dependence of the mechanism of heat inactivation of pig Lactate Dehydrogenase isoenzyme H_2M_2 FEBS Symposium 18 169-176

Tanford, C. (1968) Protein denaturation. In *ADVANCES IN PROTEIN CHEMISTRY* (Anfinsen, C.B., Anson, M.L., Edsall, J.T. and Richards, F.M. Eds) Vol 23 pp 121-282 Academic Press, New York.

Tanis, R.J. Neel, J.V., Dovey, H. and Morrow, M. (1973) The genetic structure of a tribal population, the Yanomama Indians. IX. Gene frequencies for 18 serum protein and erythrocyte enzyme systems in the Yanomama and five neighboring tribes : nine new variants. Am. J. hum. Genet. 25 655-676

Tashian, R.E. (1965) unpublished, referred to in Vessel (1965)

Tashian, R.E. and Carter, N.D. (1976) Biochemical genetics of Carbonic Anhydrase in *ADVANCES IN HUMAN GENETICS* (Harris, H. and Hirschhorn, K. Eds) Vol 7 pp 1-56 Plenum Press, New York.

Taylor, S.S., Oxley, S.S., Allison, W.S. and Kaplan, N.O. (1973) Amino-acid sequence of Dogfish M_4 Lactate Dehydrogenase. Proc. natn. Acad. Sci. 70 1790-1793

Thompson, S.T., Cass, K.H., and Stellwagen, E. (1975) Blue Dextran-Sepharose: an affinity column for the dinucleotide fold in proteins. Proc. natn. Acad. Sci. 72 669-672

Tuengler, P.and Pfleiderer, G. (1977) Enhanced heat, alkaline and tryptic stability of acetamidinated Pig heart Lactate Dehydrogenase. Biochem. biophys. Acta 484 1-8

Tuzimura, K., Konno, T., Meguro, H., Hatano, M., Murakami, T., Kashiwabara, K., Saito, K., Kondo, Y. and Suzuki, T.M. (1977) A critical study of the measurement and calibration of circular dichroism. Analyt. Biochem. 81 167-174

Ueda, N., Kishimoto, S., and Hamilton, H.B. (1974) Polymorphisms of red-cell enzymes and serum proteins (Report 1). Jap. J. hum. Genet. 19 68-69

Undevia, J.V., Blake, N.M., Kirk, R.L. and McDermid, E.M. (1972) The distribution of some enzyme group systems among Parsis and Iranis in Bombay. Human Hered. 22 274-282

Vergnes, H., Quilici, J.C., Gherardi, M., Bejarano, G. (1976) Serum and red cell enzyme variants in an Amerindian tribe. The Sirionis (Eastern Bolivia). Human Hered. 26 252-262

Vessel, E.S. and Bearn, A.G. (1961) Isozymes of Lactic Dehydrogenase in human tissues. J. clin. Invest. 40 586-591

Vessel, E.S. (1965) Genetic control of isoenzyme patterns. In *PROGRESS IN MEDICAL GENETICS* (Steinberg, A.G. and Bearn, A.G. Eds) Vol IV pp 128-175 Grune and Stratton, New York.

Wachsmuth, E.D. and Pfleiderer, G. (1963) Biochemische untersuchungen an kristallinen isozymen der Lactatdehydrogenase aus menschlichen organen. Biochem. Z. 336 545-556

Wachsmuth, E.D. and Pfleiderer, G. (1964) Aminosäurezusammensetzung von isozymen der Lactatdehydrogenase aus menschlichen und tierischen organen. Biochem. Z 340 80-94

Waley, S.G. and Watson, J. (1954) Trypsin-catalysed transpeptidations. Biochem. J. 57 529-538

Weitkamp, L. and Neel, J.V. (1970) Gene frequencies and microdifferentiation among the Makiritare Indians. III. Nine erythrocyte enzyme systems. Am. J. hum. Genet. 22 533-537

Weitkamp, L.R. and Neel, J.V. (1972) The genetic structure of a tribal population, the Yanomama Indians. IV. Eleven erythrocyte enzymes and summary of protein variants. Ann. hum. Genet. 35 433-444

Welch, S.G., Barry, J.V., Dodd, B.E., Griffiths, P.D., Huntsman, R.G., Jenkins, G.C., Lincoln, P.J., McCathie, M., Mears, G.W., Parr, C.W. (1973) A survey of blood group, serum protein and red cell enzyme polymorphisms in the Orkney Islands. Human Hered. 23 230-240

Whitt, G.S. (1969) Homology of Lactate Dehydrogenase genes: E gene function in the teleost nervous system. Science 166 1156-1158

Whitt, G.S. (1970) Developmental genetics of the Lactate Dehydrogenase isozymes of fish. J. exp. Zool. 175 1-36

Whitt, G.S., Miller, E.T. and Shaklee, J.B. (1973) Developmental and biochemical genetics of Lactate Dehydrogenase isozymes in fishes. In *GENETICS AND MUTAGENESIS OF FISH* (Schroder, J.H. Ed.) pp 243-276 Springer-Verlag, New York.

Whitt, G.S., Shaklee, J.G. and Markert, C.L. (1975) Evolution of the Lactate Dehydrogenase isozymes of fishes. In *ISOENZYMES* (Markert, C.L. Ed.) Vol IV pp 381-400 Academic Press, New York.

Wieland, Th., Duesberg, P., Pfleiderer, G., Stock, A. and Sann, E. (1962) An Dehydrogenasen gebundene umwandlungs-produkte des diphospho-pyridin-nucleotids. Archs Biochem. Biophys. Supp. I 260-263

Wieme, R.J. (1959) In *STUDIES ON AGAR-GEL ELECTROPHORESIS* Arscia, Brussels quoted in Wilkinson, J.H. (1965)

Wieme, R.J. and Van Maercke, Y. (1961 Fifth (Electrophoretically slowest) serum Lactic Dehydrogenase as an index of liver injury. Ann. N.Y. Acad. Sci. 94 898-911

Wilkinson, J.H. (1965) Lactate Dehydrogenase isoenzymes. In *ISOENZYMES* (Long, C. Ed.) pp 44-45 E. and F.N. Spon, London.

Wilkinson, J.H. and Withycombe, W.A. (1965) Organ spcificity and Lactate Dehydrogenase activity. Some properties of human spermatozoal Lactate Dehydrogenase. Biochem. J. 97 663-668

Wilson, A.C., Carlson, S.S. and White, T.J. (1977) Biochemical evolution. Ann. Rev. Biochem. 46 573-639

Withycombe, W.A. (1965) Ph.D. Thesis, University of London.

Wong, J.,T-F. (1975) Effects of temperature. In *KINETICS OF ENZYME MECHANISMS* pp,219-226 Academic Press, London.

Woodfield, D.G., Scragg, R.F.R., Blake, N.M., Kirk, R.L. and McDermid, E.M. (1974) Distribution of blood, serum protein and enzyme groups among the Fuyuge speakers of the Goilala sub-district. Human. Hered. 24 507-519

Woody, R.W. (1969) Optical properties of polypeptides in the β-conformation. Biopolymers 8 669-683

Wróblewski, F. and Gregory, K.F. (1961) Lactate Dehydrogenase isoenzyme and their distribution in normal tissues and plasma and in diseased states. Ann. N.Y. Acad. Sci. 94 912-932

Wunch, T. and Goldberg, E. (1970) A comparative physico-chemical characterization of Lactate Dehydrogenase: isozymes in Brook Trout, Lake Trout and their hybrid Splake Trout. J. exp. Zool. 174 233-252

Yoshida, A. (1967) A single amino acid substitution (Asparagine to Aspartic acid) between normal (B+) and the common Negro variant (A+) of human Glucose-6-Phosphate Dehydrogenase. Proc. natn. Acad. Sci. 57 835-840

Yoshida, A. (1970) Amino acid substitution (Histidine to Tyrosine) in a Glucose-6-Phosphate Dehydrogenase variant (G6PD Hektoen) associated with over-production. J. mol. Biol. 52 483-490

Yoshida, A. (1975) Evolution of Glucose-6-Phosphate Dehydrogenase and Hexose-6-phosphate Dehydrogenase. In *ISOENZYMES* (Markert, C.L. Ed.) Vol IV pp 853-866 Academic Press, New York.

Zinkham, W.H., Isensee, H. and Renwick, J.H. (1969) Linkage of Lactate Dehydrogenase B and C loci in pigeons. Science 164 185-187

Zuckerlandl, E. (1976) Evolutionary processes and evolutionary noice at the molecular level. II. A selectionist model for random fixations in proteins. J. mol. Evol. 7 269-312

Additional References

Greenfield, N.J. and Fasman, G.D. (1969) Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry 8 4108-4116

Laidler, K.J. and Bunting, P.S. (1973) The denaturation of proteins in *THE CHEMICAL KINETICS OF ENZYME ACTION* Oxford University Press, Oxford.

ADDITIONAL REFERENCES

Ambler, R.P. & Rees, M.W. (1959). ε-N-methyl lysine in bacterial flagellar protein. Nature 184, 56-57.

Boone, C.M. Chen, T.R. & Ruddle, F.H. (1972). Assignment of three human genes to chromosomes (LDH-A to 11, TK to 17, and IDH to 20) and evidence for translocation between human and mouse chromosomes in somatic cell hybrids. Proc. natn. Acad. Sci. 69, 510-514.

Cann, J.R. & Goad, W.B. (1968). Two or more electrophoretic zones from a single macromolecule. Ann. N.Y. Acad. Sci. 151, 638-649.

Cederbaum, S.D. & Yoshida, A. (1976). Glucose 6-phosphate dehydrogenase in Rainbow trout. Biochem. Genet. 14, 245-258.

Chang, J.Y. (1977). High-sensitivity sequence analysis of peptides and proteins by 4-NN-Dimethylaminoazobenzene 4'-isothiocyanate. Biochem. J. 163, 517-520.

Fritz, P.J. & Jacobson, K.B. (1965). Multiple molecular forms of lactate dehydrogenase. Biochemistry 4, 282-289.

Gazith, J., Schulz, I.T., Goodring, R.H., Womack, F.C. & Colowick, S.P. (1968). Multiple forms and subunits of yeast hexokinase. Ann. N.Y. Acad. Sci. 151. 307-331.

Greenfield, N.J. & Fasman, G.D. (1969). Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry 8, 4108-4116.

Hatton, M.W.C., Regoeczi, E., Wong, K.L., & Kraay, G.J. (1977). Bovine serum transferrin phenotypes AA, D_1D_1, D_2D_2, EE: Their carbohydrate compositions and electrophoretic multiplicity. Biochem. Genet. 15, 621-640.

Holmquist, W.R. & Schroeder, W.A. (1966). A new N-terminal blocking group involving a Schiff base in hemoglobin A_{1c}. Biochemistry 5, 2489-2503.

Hughes, R.C., Jenkins, W.T. & Fischer, E.H. (1962). The site of binding of pyridoxal-5'-phosphate to heart glutamic-aspartic transaminase. Proc. natn. Acad. Sci. 48, 1615-1618.

Kaplan, N.O. (1968). Nature of multiple molecular forms of enzymes. Ann. N.Y. Acad. Sci. 151, 382-399.

Kitamura, M., Iijima, N., Hashimoto, F. & Hiratsuka, A. (1971). Hereditary deficiency of subunit H of lactate dehydrogenase. Clinica chim. Acta. 34, 419-423.

Laidler, K.J. & Bunting, P.S. (1973). The denaturation of proteins. In *The Chemical Kinetics of Enzyme Action* pp.413-415. Oxford University Press, Oxford.

Law, G.R.J. (1967). Alkaline phosphatase and leucine amino-peptidase association in plasma of the chicken. Science 156, 1106-1107.

LKB Application Note 250 (1977). Analytical electrofocusing in thin layers of polyacrylamide gels. LKB-Produkter AB, Bromma, Sweden.

Lush, I.E. (1964). Egg albumen polymorphisms in the fowl: loci II and III. Genet. Res. 5, 39-49.

Margoliash E., & Fitch, W.M. (1968). Evolutionary variability of cytochrome c primary structures. Ann. N.Y. Acad. Sci. 151, 359-381.

McKenzie, H.A. (1967). Milk Proteins. In *Advances in Protein Chemistry* (Anfinsen, C.B., Anson, M.L., Edsall, J.T. and Richards, F.M. Eds.) Vol.XXII pp.55-221, Academic Press, New York.

Meizel, S. & Markert, C.L. (1967). Malate dehydrogenase isoenzymes of the marine snail *Ilyanassa obsoleta*. Archs Biochem. Biophys. 122, 753-765.

Morton, J.R. & Tobin, G. (1977). Experiments with β-chain variants of the hemoglobin of *Mus musculus*. Biochem. Genet. 15, 101-108.

Mulivor, R.A., Hannig, V.L. & Harris, H. (1978). Developmental change in human intestinal alkaline phosphatase. Proc. natn. Acad. Sci. 75, 3909-3912.

Niethammer, D. & Heunnekens, F.M. (1971). Bound TPN as the determinant of polymorphism in methemoglobin reductase. Biochem. biophys. Res. Commun. 45, 345-350.

Pantelouris, E.M. & Arnason, A. (1967). Further observations on mouse serum esterases. Comp. Biochem. Physiol. 20, 647-652.

Petras, M.L. & Martin, J.E. (1969). Improved electrophoretic resolution of some hemoglobin variants in *Mus musculus*. Biochem. Genet. 3, 303-309.

Schroeder, W.A. & Jones, R.T. (1965). Some aspects of the chemistry and function of human and animal haemoglobins. Fortschritte Chem. org. Naturst 23, 113-194.

Trayer, I.P., Harris, C.I., & Perry, S.V. (1968). 3-Methyl histidine and adult and foetal forms of skeletal muscle myosin. Nature 217, 452-453.

Ueda, N., Satoh, C., Tanis, R.J. Ferrell, R.E., Kishimoto, S., Neel, J.V., Hamilton, H.B. & Baba, K. (1977). The frequency in Japanese of genetic variants of 22 proteins II. Carbonic anhydrase I and II, lactate dehydrogenase, malate dehydrogenase, nucleoside phosphorylase, triose phosphate isomerase, haemoglobin A and haemoglobin A_2. Ann. hum. Genet. 41, 43-52.

Ursprung, H. & Carlin, L. (1968). Drosophila alcohol dehydrogenase *in vitro* changes of isozyme patterns. Ann. N.Y. Acad. Sci. 151, 456-475.

Vandekerkhove, J. & Vanmontagu, M. (1974). Sequence analysis of fluorescamine-stained peptides and proteins purified on a nanomole scale - application to proteins of bacteriophage MS_2. Eur. Jnl. Biochem. 44, 279-288.

Voogt, E. & Khan, M.P. (1978). Genetic definition of the Calcutta-1 variant of lactate dehydrogenase using man-rodent somatic cell hybrids. Abstracts of the XIV International Congress of Genetics, Moscow, 1978.

Walter, H. & Caccam. J.F. (1966). Effect of oxidised glutathione on some enzymes of erythrocytes and its relation to erythrocytic enzyme activity and electrophoretic mobility. Biochem. J. 100, 274-277.

Whittaker, R.G., Moss, B.A. & Appleby, C.A. (1979). Determination of the blocked N-terminal of soyabean leghemoglobin b. Biochem. biophys. Res. Commun. 89. 552-558.

Wieland, Th., Georgopoulos, D., Kampe, H. & Wachsmuth, E.D. (1964). Analytischer beweis der hybridnatur von isozymen der lactatdehydrogenase. Biochem. Z. 340, 483-486.

Yoshida, A. & Mega, T. (1979). Carbohydrate composition of normal and variant human alpha 1-protease inhibitors. Archs. Biochem. Biophys. 195, 591-595.

Chamoles, N. & Karcher, D. (1970a). Correlation entre l'enzymogramme classique de la lacticodes hydrogenase et son fractionnement par isoelectrofocusing en gel d'acrylamide. Clin. Chim. Acta. 30, 337-341.

Chamoles, N. & Karcher, D. (1970b). Isoelectro-focusing en acrylamide de la lacticodeshydrogenase hydrosoluble de tissus humains. Clin. Chim. Acta. 30, 359-364.

Dale,G. & Latner, A.L. (1968). Isoelectric focusing in polyacrylamide. Lancet. April 20. 847-848.

Thorstensson, A., Sjodin, B., & Karlsson, J. (1975). Separation of isoenzymes of creatine phosphokinase and lactate dehydrogenase in human heart muscle by isoelectric focusing. In *Progress in isoelectric focusing and isotachophoresis* (Righetti, P.G., Ed) pp 213-222. North-Holland, Amsterdam.

Weller, D.L., Heaney, A., Franceschi, R.T., Boudreau, R.E. & Shaw, D.E. (1973). Isoelectric focusing and study of ribosomal proteins and lactate dehydrogenase. Ann. N.Y. Acad. Sci. 209 258-280.